

Snap decisions are often unpersuasive

The consequences of the acquittal by a Los Angeles jury last week of O. J. Simpson, the US football star, would have been less perplexing if the jury had spent more time reaching its conclusion.

AFTER nine months of legal proceedings, the scant four hours spent in deciding that O. J. Simpson had not murdered his wife and a male friend were certain to give the impression that the jury had made up its mind in advance or — worse — that its members' understandable wish to escape from the irksome isolation to which the court had condemned them overrode their sense of public duty. The result is that the United States is still arguing the rights and wrongs of the case and of the verdict, which is damaging to all concerned — Simpson himself and the relatives of the victims in particular.

Meanwhile, the case has raised tricky questions about the role of technical evidence in legal trials, not only those taking place in California. The prosecution in the Simpson case, warned that defence lawyers would subject every scrap of evidence to meticulous technical scrutiny, sought to strengthen its forensic evidence by a small army of expert witnesses. For a time, in the early spring, the Simpson jury must have been better informed than any other group of lay-people about the forensic use of DNA sequences, but the 'expert witnesses' called to interpret them were often found to be less than expert by lawyers for the defence. (Prosecution lawyers later got their own back by tarring with the brush of venality the reputation of a New York forensic scientist working for the defence team.) And the Los Angeles Police Department, whose reputation is already low for other reasons, was shown to have been careless to the point of fecklessness in its handling of some samples of biological material, which may well have persuaded some jurors that none of the technical evidence could be relied upon.

Lessons

The lessons to be learned are so obvious that it is depressing that there is a need to learn them. Handguns can be stored in cardboard boxes, but the biological materials that are increasingly the substrate of forensic investigations need more care than that. And, given the ease with which the labels on laboratory samples can be switched, prosecutors can deny the suspicion that damaging samples did not originate where they allege only if the collection of samples is independently supervised, presumably (because charges will not then have been laid) by court officials. (The turning-point in the Simpson trial may have come towards the end, when a detective who claimed to have found a blood-stained glove behind Simpson's house was shown, by the defence, to be a racist bigot.) That means more time,

trouble and expense, but hardly more than the costs of the failed Simpson prosecution.

Embarrassment

Expert witnesses to the significance of technical evidence are also an embarrassment for different reasons. In adversarial systems of justice, they pop up everywhere. They deal with questions as different as whether disastrous investments may have been judged prudent when they were made, whether bridges that have fallen down were built to proper standards and the likelihood that samples of DNA derive from a particular person. Usually the experts are hired by one side or the other, often for very substantial fees. It appears not to be difficult to recruit experts with contrary opinions to the opposing sides in any trial. The result is general cynicism by jurors and the wider public about the relevance of technical evidence. (The Simpson case will also wrongly have helped to engender the opinion that the price of an acquittal is a pocket long enough to pay enough experts' fees.) Forensic science everywhere will be out of work, and its potential contribution to justice needlessly attenuated, if this state of affairs is not changed.

What is to be done? The remedy recently advocated by the British Lord Chancellor, that expert evidence should be heard in camera and an agreed report presented to the jury, will not always solve the problem; the Simpson trial showed clearly that some expert evidence is ill-considered, even partisan, meaning that the right to cross-examine expert witnesses may be an essential safeguard. A better solution, followed in some British trials of patent suits, is that courts should be enabled to hire their own adjudicating experts, although that cannot always be effective either. Yet without some means of making technical evidence effective, and persuasive outside the courtrooms in which it is heard, legal systems everywhere will be increasingly hamstrung as the world in which criminals conduct their business becomes more than ever technical.

In the Simpson trial, the jury evidently paid little attention to the technical evidence; there was not enough time. At best, the technical evidence cancelled itself out, which is presumably what the lawyers intended. The consequence is to have exacerbated the racial tensions that any verdict would have caused. (Simpson is black, as were nine of the twelve jurors.) The possibility that Simpson might have been acquitted put Los Angeles on alert for a recurrence of the riots, two years ago, following the acquittal of two white



policemen of a charge of brutality against a black passer-by. Now some sections of the white community are incensed, crying prejudice and political correctness. More deliberation by the jury would have avoided that. As things are, it is a shame that the country that has done most by legislation to promote racial harmony should now be set back by the consequences of a criminal trial. □

Tony Blair's highway

The British Labour Party's new leader has made his first mistake in two years with his superhighway deal.

THE next British government will almost certainly be formed by the British Labour Party, which held its annual conference last week at the seaside town of Brighton in southern England. Prudently, for a government in waiting, it has been circumspect in declining to say what its policies in government will be; if it has views about the proper organization of science, for example, it is not telling. That is one reason for surprise that the party leader, Mr Tony Blair, should last week have announced that he and his colleagues have made a deal with British Telecom, the privatized communications near-monopoly, under which the company will make no charge for connecting all schools, colleges, public libraries and hospitals in Britain to a broadband data network. A second reason (for being surprised) is that the deal is poorly thought through; a government-in-waiting can only damage itself with such preparation.

Thanks to US Vice-President Al Gore, access to the Internet, especially for the very young, is fast being spoken of as if it were one of the basic human rights listed by the United Nations. The educational value of such arrangements may well be as great as Gore (and now Blair) says, although the point has not been demonstrated with the clarity that would normally be required to justify the physical cost involved. Unsurprisingly, last week's conference was also loud with demands that money should be found for this or that improvement of the conventional educational system, many of which made excellent sense. Is it self-evident that access to the superhighway takes precedence over all of them — the need for more and better teachers, especially in science subjects, for example? Blair's supporters will no doubt argue that this cavil is beside the point; has not British Telecom promised to make the connections free of charge? That is a question to put to those who believe there is such a thing as a free lunch.

Objections to Blair's deal fall under two heads, commercial and constitutional. The commercial aspects of the deal do not imply unadulterated altruism on the part of British Telecom. It emerged last week, but less quickly than it might have done, that in return for connecting public institutions to the Internet and beyond, British Telecom will win the right to use whatever broadband network it installs to distribute television and other 'multimedia' signals to all who are connected to the network, either as a common-carrier or as a provider in its own right.

The company was specifically denied that right when it was privatized in 1982. Instead, the then government (whose successors are still in office) decreed that the task of 'cabling Britain' would be entrusted to a patchwork of cable television companies, that they would be free to offer telephone services to their subscribers and that British Telecom would be kept out of the supply of multimedia services at least until 2002. Although British Telecom is a partner in several of the cable companies (as are 'baby Bells' such as Nynex from the United States), British Telecom has been complaining throughout this period that it cannot be expected to install a countrywide modern communications network if it cannot get its money back by helping to meet the only established multimedia market, that for films and other video-spectacles such as prize-fights. Blair's deal will give British Telecom the prize it has been seeking all these years.

That is unwise, to say the least. Not least, it prejudices the decisions that will have to be made in 2002 or preferably sooner (see *Nature* 372, 303–304; 1994). The privatization of 1982 has succeeded in one respect: British Telecom has become much more efficient, so much so that it is well on the way to seeing off its government-appointed competitor in the field, Mercury Communications. Until AT&T won the right, earlier this year, to set up in Britain, British Telecom seemed well on the way to the restoration in private hands of the public monopoly it previously enjoyed. It is natural that it should now seek a friendly deal with the next government, one that, by offering something apparently for nothing, will make it difficult for that government to be beastly in 2002. To be fair to British Telecom, of course, the investment of £10 billion or so in a new broadband network will not be risk-free, but it is a safer gamble than some of the options for 2002. Breaking up British Telecom into smaller pieces, perhaps around the cable companies (which have already invested in broadband cable) is one of them. If Blair's deal has foreclosed that option, he will regret it.

The constitutional issue is something else. "New Labour", which is what Blair and his colleagues call their party, may be on the threshold of power, but it has plainly not yet appreciated how much power it will have if it is voted in. In the absence of a formal constitution, its power in the telecommunications field will be virtually absolute. If it were decided that the free connection of all public institutions to a broadband network is essential to Britain's future, a new government could simply make that a condition of the renewal of the licence under which British Telecom operates (and which is not part of statute law, but a commercially binding undertaking), obliging it to connect public institutions to its network free of charge. Or it could offer a similar deal to AT&T, or to the recently formed partnership between the German and French telecommunications outfits. Alternatively, it could impose a special tax on the monopoly (like the tax proposed for the privatized water utilities) and use that to pay for the connections it believes it needs. Instead, it has chosen to tie its hands in advance. That is thoughtless, and will seem naive. □

Nobel committee rewards pioneers of development studies in fruitflies

London. The 1995 Nobel Prize for physiology or medicine has been awarded to three developmental biologists — including one of the discipline's original pioneers — for research on how genes control early embryonic development in the fruitfly, *Drosophila melanogaster*. They demonstrated that a genetic approach can be successfully used as a way of studying the development of a complex organism.

The \$1-million prize will be shared between Edward B. Lewis, professor emeritus at the California Institute of Technology, Christiane Nüsslein-Volhard, from the Max Planck Institute for Developmental Biology in Tübingen, Germany, and Eric F. Wieschaus, Squibb Professor of Molecular Biology at Princeton University.

For Lewis, now 77 but still an active researcher, the Nobel Prize caps a lifetime's work in developmental genetics — he is widely considered to be the first person to have applied genetic tools to the study of embryo development — that began at the California Institute of Technology in the early 1940s.

An immensely popular scientist, Lewis was both characteristically modest and magnanimous about the award. "My contribution was not any more important than those of my co-workers, among them my wife Pamela, who isolated one of the important mutants," he says. "We just tried to push the genetics as hard as we could for 50 years, being convinced that they can help us to understand development."

Eric Wieschaus, now 48, but once a "young scientist, just thrilled to be in the lab and excited to do experiments", says that the thought he might eventually win the Nobel Prize never crossed his mind. "The real significance of the work started to become apparent to us in about 1980," he adds.

Wieschaus says that one of the lessons of the award is that it illustrates how it is seldom clear what a piece of scientific information is going to be valuable for — but often most of it turns out useful. "Looking back

we knew the experiments were important and wonderful — maybe even Nobel-worthy — but there are many such experiments and not all are rewarded."

He adds: "We were in a very privileged position in Heidelberg, able to do experiments without having to immediately justify them. Most young people today don't have the same luxury."

Nüsslein-Volhard, 52, had previously been tipped for the prize. "But when it came, it was a surprise." Her

analysing a large number of these mutants would eventually lead to major insights into how a complex body pattern is generated.

The mutated genes responsible for the transformation were found to be members of an entire family, the *bithorax* complex.

Genes at the beginning of the complex were found to control anterior body segments, while genes further down the genetic map controlled more posterior body segments. He published a key paper in 1978 (see *Nature* 276, 565–570) introducing his concepts to a wider audience for the first time.



AP

This audience included Nüsslein-Volhard and Wieschaus, both at the European Laboratory for Molecular Biology at Heidelberg. The researchers knew of a few existing mutations which disrupt the embryonic body plan. They felt a need to isolate as many mutants as possible to understand the whole process of embryonic pattern formation.

When they began their work, success was not immediately certain. But several years later, they identified a relatively small number of genes responsible for specific defects (see *Nature* 287, 795–801; 1980). "It was clear that genes somehow controlled this process," says Wieschaus.

"What we set out to do was to identify which genes." Forty-thousand random mutations were observed. Most had only minor effects on development. "But there were 150 cases or so where extraordinary things would happen," says Wieschaus.

Gerd Jürgens, professor of developmental genetics at Tübingen University and a former postdoctoral student with Nüsslein-Volhard and Wieschaus, says the three had made an outstanding contribution and were worthy winners. An "ecstatic" Kathryn Anderson, a researcher at the Department for Molecular and Cellular Biology at the University of California at Berkeley, says "this is the right combination of people and its a wonderful statement for the importance of basic research".

Peter Lawrence, a geneticist at the Laboratory for Molecular Biology in Cambridge, describes the trio as "role models for scientists of the future". The award, he adds, is a much-needed signal to science policy makers of the need to continue funding unfashionable, risk-laden, long-term projects whose result is not always in sight.

This research, Lawrence says, is the type of work that should be every scientist's bread and butter, but is becoming more and more difficult to achieve. "I am convinced that Ed Lewis would not have got a grant in our days," he says.

Barbara Cohen

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Deserving winners: Lewis (left) Nüsslein-Volhard (above) and Wieschaus (top right) have finally had their ground-breaking work on fruitfly genetics honoured.

comments echo the widely-held view among biologists that a prize in developmental biology was long overdue, particularly as the key research was performed over a decade ago.

Analysis of the fruit fly *Drosophila* dates back to the beginning of the century when the first mutations were discovered. By the 1940s it had found in Lewis an inspired young researcher willing to invest time and effort into analysing the genetic basis for a particular class of mutations, so-called homeotic transformations which cause substitution of one segment of the body with a different one. Lewis was convinced that

CREST rides a wave of popularity in Brussels

Brussels. Europe's Council of Ministers has agreed to enhance the political and strategic role of the European Commission's Scientific and Technical Research Committee (CREST), recently under fire for concentrating excessively on administrative issues.

The new functions of CREST will include responsibility for coordinating research

funded by the European Union with that of the union's 15 member states, and for the dissemination and use of research results. But the council has rejected a suggestion put forward by France that responsibility for CREST should be taken away from the commission and placed in the hands of the country holding the presidency of the council (see *Nature* 373, 276; 1995). □

Britain must tackle 'genetic illiteracy' among new doctors

London. Britain needs to increase substantially its support for the clinical applications of genetics if it is to make proper use of the information emerging from the Human Genome Programme (HGP), according to the head of one of the country's largest genetics services.

Speaking at a conference in London last week on science and medicine organized by the Department of Health, Pat Jacobs, of the Wessex Regional Genetics Laboratory in Salisbury, added that there was also an urgent need to boost the training of medical students in genetics-related issues, complaining that many doctors were coming out of medical schools "genetically illiterate".

Jacobs pointed out that, five years ago, a report from the Royal College of Physicians (RCP) had claimed clinical genetics services in Britain were on average 60 per cent understaffed. Since then, new genetic knowledge and screening techniques had doubled the work-load on such services, but with no comparable increase in funding.

"To bring it up even to the minimal level recommended in 1990, our regional authority would need to spend another quarter of a million pounds a year, and if Wessex is typical, this means spending about £4 million (US\$6.4 million) on England and Wales as a whole," said Jacobs. "This is a large sum, but it is not overwhelming, particularly when you compare it to the sums spent on the HGP itself."

Jacobs said there was "little point" spending vast sums of money on the genome project if the information it produces cannot be translated into health care because of inadequate educational and clinical resources. In particular, there was a "very urgent need" to bring staffing levels of the clinical genetics services in Britain up to the minimum level described in the RCP report.

Financial support for clinical genetics services should grow at a rate commensurate with the enormous increase in their work-load. This has resulted not only from the need to provide screening and other services directly, but also from fulfilling the huge educational role — for both the public and for medical practitioners — that has been placed on their shoulders.

"People assume that medical schools provide an excellent genetics education for the next generation of doctors," said Jacobs. "Unfortunately, this is far from true; few medical schools incorporate genetics teaching throughout the curriculum... As a result, we are going to have another generation of doctors who will be unable to cope with the so-called genetics revolution, and this also needs to be addressed urgently."

David Dickson

France asked to supply more data on N-test monitoring

Paris. The European Commission is expected formally to ask France this week to supply it with sufficient information to verify that the precautions being taken by France at its nuclear test sites at Mururoa and Fangataufa in the South Pacific are sufficient to safeguard public health.

The move follows a decision by France to deny access to some facilities to an inspection team sent by the commission to French Polynesia earlier this month. At a meeting scheduled to be held this week, the commission will establish what data it lacks, and request France to provide it.

France has accepted that the commission has the power to demand such data, and to verify control facilities, under articles of the Euratom treaty of 1957, which allows the commission to ensure that control measures taken by member states to keep radioactive emissions within certain limits are sufficient. In July, France submitted a detailed technical report to the commission on the control measures being taken in the South Pacific.

But the commission says it needs further information. Furthermore, if it decides that the tests are 'particularly dangerous', then the commission could require France to take extra control measures, under a separate article of the Euratom treaty. The French

government, however, has refused to accept the commission's competence to implement this article, on the grounds that the Euratom treaty applies only to civil, and not military, nuclear installations.

Indeed, the latter restriction means that, while the commission could eventually make public its judgement on any risks posed by the French tests, the treaty provides no mechanism by which it could require the tests to be halted.

Similarly, while the commission says it "has the option" of taking France to the European Court of Justice for infringing the treaty, should France refuse to supply all the data that the commission is scheduled to request this week, the court has no power to stop the tests. "We couldn't formally condemn France, as the tests are a military issue," says one commission official.

"The reality of the situation is that France will do whatever it wants to do", says the official. Earlier this month, for example, the commission requested France to supply it with additional data before carrying out its second nuclear test. In what could be interpreted as a diplomatic reminder to the commission of its limited powers, France proceeded with a 110-kilotonne test at Fangataufa.

Declan Butler

German states seek funding balance

Munich. In a bid to reduce regional inequalities, research ministers from the federal government and Germany's 16 *Länder* have proposed for the first time a change in the formula that determines how much an individual *Land* has to contribute to the support of a research institute in its territory.

According to the so-called Königstein Schlüssel formula, set up as part of German post-war reconstruction, the Max Planck and 'Blue List' research institutes each receive half of their funding from the federal government. The rest is shared between *Länder*, under a mechanism that has come under strain with the absorption of research institutes from the former East Germany.

At present, the host *Land* pays 25 per cent of the *Länder* share of the costs of Max Planck institutes, the remainder coming from a shared pot to which all *Länder* contribute. This limits the political influence of the host *Land* over the institutes. But it also places a heavy financial burden on *Länder* with few such institutes.

By contrast, the host *Land* bears the full *Länder* share of the Blue List research institutes. Before reunification these were relatively few. After reunification, however, the number of Blue List institutes rose from 48

to 82, with the inclusion of many institutes of the former East German Academy of Sciences. Concern over the relative weakness of quality control mechanisms has increased, while the new *Länder* in the east have been quick to complain of the financial burden.

The research ministers have now proposed, through the *Bund-Land Kommission* (BLK), which brokers agreements between federal and *Länder* governments, that the host *Land* should pay 75 per cent of the *Länder* share of the costs of its Blue List institutes. The remaining 25 per cent would come from a common pot, set up through the newly created Blue List umbrella group (AG-BL), which will arrange a formal system of evaluation of the institutes. The BLK also suggests that the host *Land* share of funding for Max Planck institutes should rise from 25 to 50 per cent.

The proposals require the approval of the finance ministers and prime ministers of all the *Länder*. Opposition is expected from *Länder* such as Thüringen, with relatively few Blue List institutes, which would still contribute the same as other regions to the central pot. This makes it unlikely that new financing mechanisms will be introduced in 1997, as the BLK suggests.

Alison Abbott

Medical bodies secure 'safe' data network

London. The British government has agreed in principle to set up a secure network for the electronic transmission of patient records. The move follows concern from the medical profession that patient confidentiality may be at risk from the growing use of 'unsecure' channels such as facsimile transmissions and, in particular, the Internet.

Last week, officials from the British Medical Association, accompanied by the heads of the various royal colleges — Britain's professional medical bodies — put the case to Stephen Dorrell, the Secretary of State for Health, for a 'secure' medical information network to handle information such as the data produced by pathology laboratories, or the transfer of medical records when patients register with a new doctor.

Dorrell is understood to have agreed in principle to their proposal, although details have yet to be worked out. Potential providers of such a network — which include British Telecom — are also believed to have been approached, and are understood to have expressed their willingness to collaborate on the project.

Several issues, however, will need to be

addressed before any encrypted network goes on-line. For example, it needs to be decided who will regulate, provide access to, and monitor security on the network. Other decisions concern who should have what level of access to information on the network, as well as the criteria for allowing entry to an individual or organization to gain entry.

The meeting between Dorrell and doctors' representatives was held in parallel with a conference on the encryption of medical records which took place at the BMA's headquarters in London last week. The BMA is expected to make a public announcement about its proposals in the near future.

The government's move coincides with a bid by the opposition Labour Party to use the potential benefits of the information

superhighway for providing medical services to justify its agreement to allow this highway to be built by British Telecom.

Speaking at the party's annual conference last week, Tony Blair, the Labour leader, singled out for special mention a proposal that the superhighway could be used to re-establish the importance of centres of medical excellence. The activities of these centres, his supporters claim, have been weakened by the 'internal market' created by the conservative government within the National Health Service.

The proposal had been put to Blair by Robert Winston, professor of fertility studies at Hammersmith Hospital in London, a leading pioneer of *in vitro* fertilization techniques (and, according to some press reports, a likely Labour nominee for a 'working peerage' in the House of Lords).

Winston claims that the internal market has been a "disaster" for centres of excellence (such as Hammersmith Hospital). By encouraging local health 'fundholders' to shop around for the most economic treatment, he says, it has discouraged such fundholders from sending patients to major research and teaching hospitals, where costs are inevitably higher.

The result, says Winston, not only makes it more difficult for such hospitals to attract patients, but may also "reduce our ability to train medical students, as we are not able to put the same quantity of clinical material in front of them". The answer, he adds, is to increase support for regional centres of excellence, and to use the superhighway to connect them with local general practitioners and other medical services.

Winston argues that such enhanced regional centres, using the superhighway to transmit "any material that can be digitized from doctor to doctor", would be in a position to take on a key strategic role in improving general standards of treatment and medical education.

"I think that it would be a very good thing for medicine, but it needs the stimulus of a government prepared to do things that are currently held back by the philosophy of the internal market," says Winston.

● Britain's former Archbishop of York — the second-most senior Church of England official — Lord Habgood, has criticized the Internet as a medium in which "truths and principles" are being replaced by an "endless succession of human opinions".

Lord Habgood, a trained biologist, who retired as archbishop in the summer, said in the annual Priestland Memorial Lecture that the volume of information on the Internet was in danger of disorienting people.

"The accumulated wisdom of past generations," he said, is being replaced by a "mad scramble for personal fulfilment".

Ehsan Masood & David Dickson

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**Winston: medicine can
use the superhighway.**

Universal Pictorial

HIV compensation package under offer

Tokyo. Over 200 Japanese haemophiliacs infected with HIV by contaminated blood products will each receive ¥45 million (US\$450,000) in compensation from the government and pharmaceutical companies if a compromise proposal announced last week by the Tokyo and Osaka district courts is accepted.

The recommendation follows years of litigation that began in 1989 when a group of infected haemophiliacs brought the first 'HIV suit' claiming that five companies that had imported the blood products into Japan — Green Cross Corporation, the Chemo Sero Therapeutic Research Institute, Baxter Ltd, Bayer Yakuin Ltd and Nippon Zoki Pharmaceutical Company — as well as the government that approved their sale, were guilty of negligence.

By agreeing to the plan, the government would avoid a possible court ruling that it was negligent in allowing contaminated blood products to be marketed in Japan for several years after heat-treated products were available. The government's acceptance of the plan would not set a legal precedent. But Shinichi Tokunaga, a lawyer representing the plaintiffs in Osaka, says that it would strengthen the hand of future litigants.

The haemophiliacs' group alleges that the government and the companies allowed potentially infected products to be

used, despite knowing they might be dangerous. As in France, where government officials have already been jailed because they delayed introduction of heat-treated products (see *Nature* 375, 349; 1995), Japan did not allow the use of coagulants treated with heat to kill HIV and other viruses until 1985, more than two years after they were approved for use in the United States.

In June 1983, a division of the Ministry of Health and Welfare's pharmaceutical affairs bureau set up an AIDS study group, headed by Takeshi Abe of Teikyo University, to advise on blood products policy. This advised the government to withhold approval of heat-treated products until clinical trials were completed in Japan. In the ensuing two years, up to 2,000 haemophiliacs were infected.

The settlement proposal calls for each plaintiff to be paid ¥45 million, with the government meeting 40 per cent of the cost. The plaintiffs had requested ¥115 million for each plaintiff, as well as apologies from the government and the companies. Toshihiro Suzuki, leading counsel for the plaintiffs in Tokyo, promised to continue to fight until they received an unequivocal apology from the ministry, which is refusing to speculate on whether it will accept the plan, or offer compensation to all those infected.

Steven Barker

Austrian universities come under scrutiny...

Munich. Austria's biomedical research community has come in for harsh criticism in a report by an international review committee, commissioned by the Austrian Biochemical Society and presented to the ministry of science and research last week.

The report says that the standard of biomedical research in Austria is, on average, well below that of other European countries of similar size and wealth, such as Sweden, the Netherlands and Switzerland. It puts most of the blame on Austria's universities, where most such research is carried out and which can give tenure to scientists without requiring proof of research competence.

The review committee, chaired by Fritz Paltauf, a biochemist at the Technical University in Graz, was organized by the European Molecular Biology Organization. It says that while several centres carry out "excellent work", the scientific output of many research groups is poor, and criticizes an apparent lack of concern in the academic world that such groups are still funded.

The prime cause of this poor performance, according to the report, is Austria's "misguided" policy for academic appointments, which has had a "devastating" effect on many institutes. Tenured positions are too often made on non-scientific criteria such as age. The review panel claims this blocks opportunities for ambitious young researchers, already hampered by a law preventing temporary appointments for professors and assistant professors.

University traditions also leave the research community split up into groups that are too small to compete international-

critically, with larger grants given to strong research groups.

The Austrian Biochemical Society says it "largely agrees" with the report's conclusions, but blames the absence of non-university research institutes in Austria, in addition to university procedures, for its failings in biomedical research.

But the problems identified by the review panel are not confined to biomedical research. Austria's 18 universities, traditionally tightly controlled by the science and research ministry in Vienna, are attempting to implement a new law, passed in 1993, that gives them extensive administrative and financial autonomy.

The law requires that procedures be set up to ensure that successful researchers are allocated an appropriate proportion of university resources. But the implementation of these procedures remains slow in the face of considerable resistance from the universities. Meanwhile, a further draft law, drawn up by the research ministry to allow temporary positions for professors, continues to meet political opposition, particularly from trades unions.

Helmuth Ruis, a professor of biochemistry

at the University of Vienna's Biozentrum who has long argued for a more selective distribution of research money and is a driving force behind the setting up of the review panel, says he hopes that its conclusions will help to convince the government it must address the university system's shortcomings.

Outside the universities, however, there are some encouraging signs of change. Most public funds for basic research are distributed through the national agency, the *Fonds zur Förderung der Wissenschaftlichen Forschung* (FWF). This has long been criticized as operating on the 'Gießkanne' or 'sprinkler' principle, with research money distributed to weak and strong alike in 'drops' too small to be effective.

This year, however, the FWF has raised its rejection rate for grant applications from 25 per cent to 40 per cent, and the length of grants from two to three years. The agency has also just launched a programme giving young scientists five years of generous funding, averaging ÖS2.5 million (US\$240,000) per year, to set up independent their own research groups. Ten scientists will be funded in this way each year. **Alison Abbott**

... as Japan backs fixed-term posts

Tokyo. In a significant break with tradition, Japan appears poised to take its first tentative step towards introducing limited-term appointments — rather than lifetime employment — for university staff.

The change has been recommended in the interim report of a committee set up by the University Council, an advisory body to the Ministry of Education, Science, Sports and Culture (Monbusho). It is aimed at encouraging better research and teaching, and could eventually lead to a radical reform of the whole university system.

Winning acceptance of the idea of limited term appointments "took a long time to achieve", says Akito Arima, vice-president of the council. Arima is president of the Institute of Physical and Chemical Research (RIKEN), as well as a former president of Tokyo University, and headed the council committee responsible for writing the interim report.

Nevertheless, Arima admits that he wanted "a more decisive decision" at this stage on the reform of employment practices, and says he still sees a long road ahead before the changes recommended in the report — to be followed by a more substantial final report in about a year — are implemented.

Reflecting conflicting views in the council itself, the report suggests that individual universities should be allowed to decide whether to introduce limited-term appointments, to set their duration and to specify

the conditions under which they can be renewed. As such, the recommendations are "very weak", admits Arima, who has been campaigning vigorously for years for reform of Japan's university system.

Along with limited-term appointments, the council recommends a system of assessing university staff that would operate before a contract is renewed. The report gives no details of how such assessments should be carried out, or how the results should be applied. But it would include an assessment both of teaching — possibly by students — and of research output.

Introducing limited-term appointments will make it necessary to rewrite Japan's labour laws. These stipulate that government employees, including national university staff, can be employed only on a permanent basis. "My main aim is to change the law," says Arima. He points out that a university could at present be successfully sued for trying to dismiss a member of staff.

Some universities have already managed to reach agreement with some staff to limit the length of appointments. At Kyoto University's Research Institute for Fundamental Physics, for example, full and associate professors are usually limited to terms of 5 to 10 years, and research assistants to 3 to 6 years under an informal 'gentleman's agreement'. Changing the law would put such a system on firm legal footing.

But the new law could not be applied ►



ly. The panel does not criticize the level of research funding. But it points out that the money tends to be spread among too many groups rather than being concentrated on the stronger ones.

A top priority, says the review panel, is that universities should be required to evaluate the research competence of scientists being offered tenure. It also recommends that grant applications be assessed more

retroactively. All existing university staff would therefore retain the right to lifetime employment. Unsurprisingly, therefore, most senior university staff support the councils' recommendations, while many young researchers who have been waiting in line for permanent positions — often for years — are opposed to them.

To ease the situation of young researchers, the coalition government has, at Arima's urging, agreed to increase the number of postdoctoral fellows in Japan from the present level of 4,000 to 10,000 by the year 2000. That will help the many so-called 'over-doctors' (a Japanese term derived from English) who have PhDs but no permanent post and who continue doing research by supporting themselves with part-time jobs.

To pay for this, both Monbusho and the Science and Technology Agency have applied for extra funds next fiscal year (which begins on 1 April), and Monbusho plans to boost the salary for fellowships from ¥280,000 (\$2,800) to ¥400,000 a month to make them more attractive.

At the same time, Arima is urging the Japanese government to stop employing young scientists as research assistants, a position that provides lifetime employment. Instead, he would like it to appoint technicians to such posts, thereby helping to ease the chronic shortage of technical staff in universities. He hopes this policy will be adopted next year, "provided that they [young researchers] don't kill me first".

David Swinbanks

Europe to strengthen links on marine and polar projects

Paris. Scientists from 30 institutes in 17 European countries have agreed to set up two new bodies aimed at improving the coordination of marine and polar research in Europe. The European boards for marine and polar science, as they are known, will be established under the auspices of the European Science Foundation (ESF), based in Strasbourg, France. Each will have 20 members, overseen by a joint executive committee, and will be administered from the ESF.

The marine science board will be chaired by Daniel Cadet, deputy director of the Institut des Sciences de l'Univers of the Centre National de la Recherche Scientifique (CNRS). Barry Heywood, director of the British Antarctic Survey, will chair both the polar sciences board and the executive committee overseeing the two boards.

According to Heywood, the reason for setting up the boards, agreed at a meeting in Paris last week, is the need "to find quick answers to very important scientific problems at a time when budget resources are shrinking and research equipment is getting more expensive".

To achieve this, the meeting concluded that Europe needs large programmes lasting at least ten years, and costing more than ECU50 million (US\$61 million). Until now,

most marine and polar programmes in Europe have tended to be national programmes lasting between one and three years, and the large programmes identified could not be afforded by one nation alone.

The first programme for which the boards will seek support is on climate change, the European Project for Ice Coring in the Antarctica (EPICA). Estimated to cost ECU50–ECU60 million, this will investigate whether the findings of ESF's Greenland Icecore Project (GRIP) — that the Earth's climate has been stable for around 10,000 years, but fluctuated violently before that — are a regional or global phenomena.

Other so-called 'grand challenges' identified at the meeting include a programme forecasting physical, chemical and biological processes in oceans and coastal seas over timescales ranging from seasons to decades, a project to map the Arctic Ocean and a study of the deep sea floor.

Half the funding for such programmes would come from European Union (EU) research funds, in particular, the Marine Science and Technology (MAST) programme — which has a budget of ECU228 million for the period 1994–98 — and environment programmes. The other half would come from national research agencies.

Proposals for EU funding would be required to compete with other bids under standard research grant procedures. Nevertheless, the European Commission, which has been involved in the creation of the boards from the outset, is enthusiastic about the prospect of large cooperative programmes. "The scientists took this initiative, and we are keen to support them," says one commission official.

Heywood says the boards are willing to tailor proposals to the commission's requirements, pointing out that many of the boards' members are also members of the programme committees. But he emphasizes that the boards will proceed modestly. "We want to be pragmatic", he says, with each national group first identifying its own interests and then discussing possible collaboration with others. The boards' role will be primarily to implement agreed projects, he says, not to act as a talking shop. "We need to obtain street credibility, in particular with national governments."

One aim of the boards will be to seek better use of Europe's existing research fleet of 20 large vessels, as well as costly facilities such as submersibles and satellites. Ultimately their goal is to provide Europe with a single voice for marine and polar science in negotiations with the European Commission and other international bodies.

Declan Butler

Paris pendulum swings back into action

Paris. **France's annual science festival — Science en Fête — got off to a swinging start last week when the original Foucault pendulum (right) oscillated under the majestic dome of the national mausoleum, the Panthéon, for the first time since Léon Foucault used it to demonstrate Galileo's theory that the Earth turns on its axis.**

Foucault's original pendulum bob — a brass-plated lead ball, 38 cm in diameter, weighing 28 kg — was taken from its home in the Conservatoire National des Arts et Métiers for the reconstruction. It was attached to the apex of the Panthéon dome, 67 metres above the ground, by a steel cable, and after its release by François Bayrou, France's Minister of Education, soared back and forth across the Panthéon, taking 16.5 seconds to complete each swing.

The science festival, which this year includes more than 1,800 scientific events, is unlikely to create

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the same stir as Foucault's first demonstration of his pendulum, which proved an immediate success with the public and was subsequently reproduced in many other cities. Recognition of Foucault — an autodidact who also invented the gyroscope — by the scientific community took longer; he was admitted to the French Academy of Sciences only in 1865, three years before his death.

D. B.

US radiation report fails to satisfy critics

Washington. Groups representing individuals who were the subject of human radiation experiments in the United States after the Second World War have reacted angrily to the main findings of the Presidents' Advisory Committee on Human Radiation Experiments, which reported to President Bill Clinton last week.

The groups, broadly represented by a body known as the Task Force on Radiation and Human Rights, have attacked the report — prepared by a panel chaired by Ruth Faden of Johns Hopkins University in Baltimore, Maryland — for failing to recommend any medical follow-up to the experiments, or compensation for subjects who are unable to prove that they experienced physical harm.

Geoffrey Sea, a health physicist based in Oakland, California, and the West Coast coordinator of the task force, describes the report as "absolutely inadequate". Similarly, David Egilman, an assistant clinical professor at Brown University, Rhode Island, an outspoken critic of the radiation

experiments, says that the advisory committee did "an abysmal job".

It was complaints from members of the task force that initially led Hazel O'Leary, the energy secretary, to initiate the process that culminated in last week's report. The task force is now planning to formally reject an apology that was issued by Clinton when the report was released (see *Nature* 377, 374; 1995).

Clinton had offered "sincerest apologies to those citizens, their families and their communities" on behalf of the US government. But the activists want cash compensation for thousands of people unknowingly subjected to the experiments.

They also want all such experimental subjects to be formally notified of what happened, and to be offered medical follow-up. The advisory committee ruled that cash compensation would be morally out of proportion to the original wrong, and that it was now too late for any useful follow-up.

Some of the group's concerns are shared by politicians who have supported the

subjects' cause, such as Senator John Glenn (Democrat, Ohio). After the report was released, Glenn called for "a limited medical monitoring programme" to follow up the experiments.

Responding to the committee's finding that existing protections for human research subjects are "seriously deficient", Clinton asked all the federal agencies involved to review procedures for protecting the subjects of experiments, and to report back within three months to the planned National Bioethics Advisory Commission (NBAC), which he also announced last week. The NBAC's members have not yet been named.

The Secretary of Health, Donna Shalala, whose department conducts most US research on human subjects through the National Institutes of Health, said that she accepted the criticism of existing protective procedures. "We accept the fundamental premise that significantly more needs to be done" to protect human subjects, she said.

Colin Macilwain

Protest over spent fuel shipment highlights storage crisis

London. Anti-nuclear activists last week attempted to block the shipment of 52 spent fuel rods from a research reactor in Germany to a reprocessing facility at the UK Atomic Energy Authority's Dounreay plant in Scotland.

Protesters from the environmentalist group Greenpeace managed to delay the shipment from the German port of Bremerhaven for several hours by chaining themselves to containers holding the fuel rods. A demonstrator was arrested two days later when the shipment, from the Hahn Meitner Institute (HMI) in Berlin, was unloaded in the port of Scrabster near Dounreay.

The decision to send the spent fuel to Scotland — rather than the United States, where it originated — follows similar protests last year from local groups in South Carolina, where the fuel rods had previously been scheduled to be reprocessed under a US-German agreement.

The latter agreement followed an earlier decision taken by the United States in 1978, under which it promised to take back spent, high-enriched uranium (HEU) fuel of US origin — concerned that this could be used to produce plutonium for weapons — from countries promising to convert their reactors to low-enriched uranium (LEU).

French nuclear tests: A recent article (see *Nature* 377, 186; 1995) on an exchange in the French press about the significance of the nuclear tests in the South Pacific should have mentioned that this exchange was published in the newspaper *Libération*.

A court decision in South Carolina forced a delay in any decision over the eventual fate of the fuel rods until the completion of an environmental impact statement by the Department of Energy (DoE) (see *Nature* 376, 716; 1995). The outcome of the assessment will be announced later this month.

But the delay has left research reactors throughout Europe near the limits of their storage capacities. It was this crisis that forced the HMI to sign the Dounreay contract.

Despite local opposition, the UK Department of Trade and Industry (DTI) is keen for the United States to fund reprocessing at Dounreay to keep the plant, a likely victim of the virtual collapse of interest in fast breeder reactors, in business over the next ten to fifteen years.

The US impact statement is expected to consider disposal at Dounreay as an alternative option to that preferred by the US government, namely reprocessing at the DoE's facilities in North Carolina. But Mike Townsley of Greenpeace points out that, while three major public consultations have taken place in the United States about what should be done with the waste from reactors using HEU, "nobody has bothered to ask the Scottish people what they want".

Local concern in Scotland is focused on the possible increase in radioactive emissions from Dounreay resulting from any new contracts. One reprocessing plant is already engaged at full capacity in decommissioning the reactor core of Dounreay's Prototype Fast Reactor (PFR). The small shipment from Germany, along with a much smaller

one from a Scottish reactor, will require the reopening of a second plant, the Materials Testing Reactor (MTR) reprocessing plant, in January.

According to Lorraine Mann, of the protest group Scotland Against Nuclear Dumping, the United States, under the terms of its nuclear anti-proliferation policy, is prevented from endorsing the opening of such a reprocessing facility, as that would keep the plutonium in circulation.

Anti-proliferation groups in the United States, such as the Nuclear Control Institute, remain concerned that European deals such as that between the HMI and the UK Atomic Energy Authority may encourage trade in weapons-grade fuel.

Meanwhile participants attending a meeting in Paris last month endorsed a letter to President Clinton expressing their concern that the 1978 agreement may now be in danger of collapse. The letter cites in particular the apparent lapsing of the United States' agreement to take back spent fuel, and the suspension of US support for the development of high-density LEU fuel at the Argonne National Laboratory.

The letter diplomatically avoids direct mention of the planned FRMII reactor, the first designed to burn HEU since the start of the 1978 agreement. At the Paris meeting, representatives from Argonne put forward an alternative design that would allow high-density LEU fuel to be used in the reactor. But the Technical University of Munich has rejected suggestions of a redesign.

Ayala Ochert & Alison Abbott

Row erupts over evacuation plans for Mount Vesuvius

London. Members of an Italian government commission given responsibility for setting up a contingency plan for the possible eruption of Mount Vesuvius have fallen out over the final report, which one member describes as being over-ambitious and scientifically flawed, and thus unworkable.

Giuseppe Luongo, professor of physical volcanology at the University of Naples, says the plan claims that volcanologists can predict an eruption within 20 days. But he argues that the plan is based on flawed data and was drawn up without peer review, and with virtually no input from the local population.

"I am sad to see millions of taxpayer dollars being wasted on producing [this plan], and also regret that its authors appear ignorant of current scientific literature on the subject", says Luongo, a former director of the Vesuvius Observatory. "I would like to know who are these scientists who think they can issue a volcanic eruption forecast 20 days in advance. In my experience of similar volcanoes — for example, Mount St Helens in 1980 and Pinatubo in 1991 — scientists are very reluctant to issue such forecasts without objective data."

The plan, issued by the Department of Civil Protection at the end of last month, assumes that volcano monitoring stations will remain operational up to a few days preceding an eruption. Luongo says that it apparently makes no allowances for false alarms, and fails to outline a clear chain of command. Furthermore, the plan was developed using the 1631 eruption of Vesuvius as a template, and Luongo says such an assumption could be a mistake as that eruption, which killed several thousand people, remains highly controversial among volcanologists.

But Lucia Givetta, director of the Vesuvius Observatory and a member of the commission, disagrees. She says that the volcano has always emitted distinct, characteristic precursor signals before an eruption. The most recent eruption — which took place during the Second World War in 1944 — was accurately predicted by one of her predecessors, Giuseppe Imbi, "using just one instrument", she says. "Unfortunately, the soldiers didn't listen to him, and no counter-measures were taken."

Givetta says the commission's data are no secret, as they are unchanged from the previous commission's report in 1992. "It has been extensively discussed and debated. This plan was not just born yesterday, it is

the result of almost five years' hard work."

Nevertheless Luongo, despite being the only member of the five-person commission to vote against the plan, claims to have the support of many volcanologists at Italian universities who, he claims, are unable to speak out openly. One who did so — Flavio Dobran, a former professor of volcanology at the universities of Rome and Pisa — claims to have had his research grant terminated after publishing a paper (see *Nature* 367, 551–554; 1994) that defied the orthodox

IMAGE
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Twin peaks: the emergency plan assumes a 20-day warning.

government view by suggesting that an outpouring from Vesuvius could destroy everything within a 7-km radius, an area occupied by 1 million people.

Dobran, who has now set up his independent research group, the Association for Global Volcanic and Environmental Systems Simulation (GVES), points out that the plan apparently fails to prioritize categories of evacuees, and does not explain how different communities are expected to reach emergency transport stations in the event of seismic activity and ground deformation.

The plan assumes 600,000 people would be evacuated within a week, on 40 trains running daily. The plan appears to assume that trains and buses will run according to a fixed timetable. "This does not even happen under normal circumstances," he points out.

Dobran says the government's plan is preoccupied with trying to predict an eruption, when it should be concerned with disaster management and risk communication. He is now engaged in a project called Vesuvius 2000, a public education/consultation exercise. He is thus critical of plans to evacuate people in risk zones to destinations at great distances from Vesuvius.

"Evacuating 600,000 people is no joke," he says. "It raises many questions, such as the threat to a lifestyle, culture and centuries of traditions. The population should be educated in the different risk scenarios posed by a potential eruption and then be given the choice to decide how it wants to react." But Givetta says these are political issues and should not concern scientists. "Our job is to get the science as accurate as possible. The rest we leave to politicians. **Ehsan Masood**

'Ig Nobel' ceremony reaches new heights and moves to Harvard

Boston. The Fifth Annual 'Ig Nobel' Prize Ceremony, held on 6 October, was a night of many firsts. The proceedings were televised live via the Internet, a feat never previously attempted. It featured the world premiere of the "Interpretive Dance of the Nucleotides", featuring the Nicola Hawkins Dance Company and five Nobel prizewinners.

Sheldon Glashow and harpist Deborah Henson-Conant performed a second world premiere, *Cymbalic Duet in B[♯] Major with Psychological Overtones*. And in yet another first, the ceremony was held at Harvard University rather than at neighbouring Massachusetts Institute of Technology (MIT), site of the four previous 'Igs'.

The change of venue was due to a rift earlier in the year between Marc Abrahams, editor of the *Annals of Improbable Research* (AIR) — a sponsor of this year's and last year's 'Ig' ceremony — and the MIT Museum, the previous 'Ig' co-sponsor which had also been publishing the *Annals*.

The falling-out was surprising in view of the gala celebration at the museum in December 1994 of the release of AIR's first issue, when many were saying that MIT was "the perfect home" for a magazine such as AIR and the associated award ceremony.

Both sides were tight-lipped about the controversy, which apparently revolved around the failure of Abrahams and the museum to agree on a contract for the continued publication of the magazine. MIT sources also claim that the museum staff had grown weary of the amount of time spent on the annual Ig event.

Abrahams has since published and edited the magazine on his own, finding a new home for the Igs — Harvard's Lowell Lecture Hall — with help from Glashow and fellow Nobel laureates Dudley Herschbach and William Lipscomb. Two student organizations — the Harvard Computer Society and *Tangents* (the Harvard-Radcliffe Mathematical Bulletin) — agreed to co-sponsor the event.

The 1995 Ig festivities, built around the theme of DNA, contained the usual assortment of jokes, outbursts and 'spontaneous combustion'. The five Nobel prizewinners participated directly, while six others sent taped messages to the winners and audience.

Why do these distinguished scholars subject themselves to the often embarrassing moments that make up the Igs? "This is a chance to put some fun back into science," Herschbach says. "Scientists do have a lot of fun, and it's important that the public, and especially young people, realize that. We aren't just a bunch of super-nerds with no sense of humour." **Steve Nadis**

J. E. Guest/FIPA

Impact of climate change

SIR — I should like to comment on your report headed "Developing countries dispute use of figures on climate change impacts" (*Nature* 376, 374; 1995) about a meeting of Working Group III of the Intergovernmental Panel on Climate Change (IPCC).

The terms of reference approved by governments for the working group require it to assess the socio-economic literature related to, among other topics, climate change damage, mitigation costs and applicability of cost-benefit analysis to analysis of climate change. The story implies that Working Group III "put a value on a death in a developed country". This statement is incorrect. The figures cited in the story are drawn from the peer-reviewed literature, not calculations by Working Group III, which has not and will not carry out new calculations.

The article also refers to "damage caused by a doubling in carbon dioxide concentrations (1.5–2 per cent)". The working group report points out that the literature contains only a few estimates of climate change damage. The values cited in the story are qualified as 'best guess' central estimates for a doubled carbon dioxide concentration and equilibrium climate change, on an economy with the same structure as the present world economy.

The damage cost estimates in the literature include a value for the increased risk of mortality due to climate change. Studies that estimate the damage caused by climate change at a regional level have used estimates of the value of a statistical life for the different regions. Those values are substantially higher for industrialized regions than for developing regions. The Working Group III report also describes the effect of adopting a uniform value of a statistical life for all regions. Finally, the report also points out that, in formulating climate change policy, decision-makers may well wish to assign different equity weights to the value of various damages, including the risk of mortality, in different regions.

The working group's draft 'summary for policy makers' presents two estimates of mitigation costs for greenhouse gases. First, it says that energy efficiency of perhaps 10 to 30 per cent of current consumption can be realized at negative to slightly positive cost. It also points out that estimates of the cost of stabilizing carbon dioxide emissions at 1990 levels for several decades beyond 2000 in OECD countries vary widely, but 'top-down' models project that they may ultimately exceed 1 to 2 per cent of gross domestic product.

When your story says that the "IPCC committee had calculated that slowing

down global warming could be more expensive than merely paying for the damage caused", it is apparently comparing the 'best guess' estimate of damages for a doubled carbon dioxide equilibrium climate with the 'top-down' model estimate of the cost of stabilizing carbon dioxide emissions in OECD countries for several decades beyond 2000. The costs and damages compared relate to completely different situations. The comparison is simply not valid.

I believe that the draft report clearly recognizes the limitations of benefit-cost analysis for climate change policy. The inference that the Working Group III draft report presents a cost-benefit analysis that suggests that little need be done to slow warming is completely inaccurate.

James P. Bruce

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SIR — Your news item reports me as saying that developing nations have so far failed to propose a single workable alternative to Western cost-benefit models.

In fact, what I told your reporter was that the available literature does not contain information on methods of putting a value on life other than what is reported by Working Group III of the Intergovernmental Panel on Climate Change (IPCC); almost all the available literature on the subject is to be found in the developed world.

N. Sundaraman

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Hidden meaning

SIR — I was amused to see the word "tucarezol"^{1,2} used as the name of a drug with potential in the treatment of chronic infectious disease and cancer.

The British authors unconsciously make a pun in Japanese.

In Japanese, *tucare* means "fatigue", and *sol* means "shave" (or, in the case of *tol* means "remove"). "Tucarezol" therefore means "reduce fatigue".

Tomoyuki Hisa

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1. Rhodes, J. *Nature* 377, 71–75 (1995).
2. Shearer, G. M. *Nature* 377, 16–17 (1995).

Italian reforms

SIR — There has been much discussion, both in Italy and abroad, about the way in which candidates are appointed to university posts in Italy.

Good candidates are often passed over, and recourse to the law is unproductive because of the delays and the costs involved. The minister for universities and research, Professor Giorgio Salvini, is reported to be preparing reforms, but many of us have little confidence that they will be successful because they do not take sufficient account of the principal issues.

Italian universities need to share the standards of efficiency, honesty and justice demanded by international competition and Italy's cultural tradition. The chief problem with Salvini's proposed law is that it entrusts the enforcement of the new rules to the same people who are responsible for the present state of Italian universities.

In particular, as I understand it, the proposed reforms ignore the plight of those who have already been passed over; this is damaging to the universities and discouraging to the new generation. The new law also provides for about 50 per cent more certifications than the number of positions available, which will be valid for two years after the job competition has taken place, giving those already certified an advantage.

There are many other grave defects in the proposed reforms. What Italy needs, by contrast, is a system in which both the duties of professors at different career levels and promotion from one level to another are decided honestly and on merit alone. Changing the details of the present system will not remedy its glaring faults.

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Wrong place

SIR — I was concerned to read in your article "More precise solar-limb light-bending" (*Nature* 377, 11; 1995) that Eddington and his associate Crommelin had taken themselves off to the western Pacific to observe the total solar eclipse of 29 May 1919. Hitherto I was under the impression that Eddington had gone to Principe off the West African coast, and Crommelin had gone to Sobral in Brazil.

If your article is correct then there are grave implications for the gravitational bending of light and I am surprised that the standard errors of the measurements were so small.

Colin Davies

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Preparation for university

SIR — As a college (university) representative on the governing body of a secondary school. I recognize only too easily the educational problems set out in your leading article (*Nature* 377, 2; 1995).

It is convenient for universities to recruit students who have already covered much of the material taught in the first year of a three-year course, possibly releasing more time for research; and, naturally enough, secondary schools whose academic success is measured by the number of sixth-formers going on to university fall in with what the universities appear to want; but if one asks dons, teachers, parents, pupils and educationists what is desirable, nearly all opt for less specialization and a smaller workload at sixth-form level for the reasons that you so clearly set out.

One undesirable effect is to deflect able students who could become good scientists into the arts as offering more scope for creative thinking, thus avoiding the long march towards the front line of science burdened by knapsacks crammed with unassimilated information. As you say, other countries produce good scientists without inflicting a specialized education upon them at too early an age: for instance, John Enders was reading English at Harvard when he became interested in microbiology.

What is now needed is a conference involving headmasters, vice chancellors, educationalists — and why not some able sixth-formers? — to work out a more sensible policy which would then be tried out in some schools and universities willing to take the risk. Mrs Gillian Shephard is just the right person to convene it.

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SIR — There is nothing "strange" about the view that A-levels have become easier, as the view follows easily establishable facts. Joe Ruston (letter, *The Times*, 24 August 1994) pointed out that candidates prepared for the 1994 A-level examination in physics could have attempted questions accounting for only 22 per cent of the marks in the 1974 paper. By contrast, candidates prepared for the 1974 paper could have answered all of the questions in the 1994 paper (disposing of the argument that the syllabus has broadened but not declined in standard). Indeed, candidates prepared for the 1974 O-level could in fact have answered questions amounting to 40 per cent of the

marks of the 1994 A-level. If discussions on quality in science education, and its change over time, are to have any credibility, it would be helpful if they would take these facts into account.

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Cost of psychiatric care

SIR — It was stated in a recent leading article that "psychiatric hospitals are expensive to staff and run" (*Nature* 376, 714; 1995). This is a common misconception.

A recent paper published by the Institute of Psychiatry and the University of Kent provides evidence that "good community care is not a cheap option" (A. Hallam, *Mental Health Research Review* 2, 29–32; 1995). After a nine-year study of the closures of two long-stay psychiatric hospitals in North London (Friern and Claybury), the paper concludes that the costs of hospital and community care are essentially the same, but that the mean cost per patient will rise as the final cohorts of "difficult to place" patients are discharged. Furthermore, the final mean patient cost may continue to increase because "as the psychiatric institutions disappear, inpatient episodes usually take place in acute, general hospitals, at a cost which may be four or five times greater [than in a psychiatric hospital]". The public perception of mental illness is already coloured by plausible but unsubstantiated beliefs. In the brave new world of "evidence-based medicine", *Nature* is an old hand at sifting evidence, and should therefore be careful not to advance such unsubstantiated beliefs.

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Greeks overseas

SIR — We represent a group of Greek scientists around the world who have not yet served our 18-month national service, pursuing our career in science with the encouragement of the state through continuous exemptions from the armed forces. There are probably 30,000 of such males in the 25–35 age range, mainly in Europe and the United States.

We are unable to return to our homeland without facing charges and doing national service for at least a year and a half, a devastatingly long time in the career of a research scientist. Consequently, we are not able to visit our fami-

lies or search for academic and research positions in Greece. Our inability to return home creates serious personal problems with repercussions for a large segment of Greek society. The government announced the implementation of a new law in October 1994 that in principle would allow us to serve partially and resolve the present difficulties. The current law, which allows the further exemption of "distinguished" scientists abroad, is not properly implemented, because the reviewing procedures are carried out by military personnel and not by scientific referees.

Many of us, apart from scientific responsibilities, have family obligations that force us to move abroad. We appeal to the Greek authorities to accelerate the process for the implementation of the new law and to provide us with the opportunity to pay our dues to Greece without severe setbacks in scientific research and our personal lives. The brain-drain could thus be halted.

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Theodoros G. Kallitsis (*University of California, Davis, USA*); **Panagiotis Kouklis** (*HHMI, University of Chicago, USA*); **Panos Kudumakis**, *King's College, London, UK*;
Stefanos Sarafianos (*Rutgers University and Center for Advanced Biotechnology and Medicine, Piscataway, New Jersey, USA*); **Michel T. Semertzidis** (*Credit Commercial de France, Paris, France*); **Manolis Tomadakis** (*Florida Institute of Technology, USA*)

Salk and science

SIR — Renato Dulbecco's otherwise excellent obituary of Jonas Salk (*Nature* 376, 216; 1995) is marred by its conclusion. Dulbecco writes that Salk "...received no recognition from the scientific world.... The reason is that he did not make any innovative scientific discovery. The fact that a fundamental advance in human health could not be recognized as a scientific contribution raises the question of the role of science in our society.... It is true that [Salk] did not contribute any technological advance; but is science only technology?"

I think this has matters exactly backwards. Salk made an extremely important technological advance: he showed how to apply scientific knowledge to make a polio vaccine. Science, however, is not technology. Rather, it is the discovery and systematization of knowledge about the natural world. This, *inter alia*, makes technological advances such as the polio vaccine possible.

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Fraud and hoaxes in science

William H. James

The recent spate of moral condemnation of fraud in science reflects the conservative nature of scientists; fraud, like error, is a normal part of science and cannot be legislated away.

I SHOULD like to offer some qualifications to the current crusade against scientific fraud. It seems to me that scientists are being po-faced. The metaphor of a few rotten apples (in a barrelful of sound ones) is deceptive: it implies that we may believe any paper that is not by a few identified fraudsters. I suggest that the overstrident urge to condemn scientific malpractice is due to the unadventurous nature of scientists today, and their failure to understand the nature of science — to appreciate that judgement is required in assessing the validity of scientific claims.

Scientists are selected by examinations that favour vertical over lateral thinking; they have to demonstrate their docility by learning the curriculum (nine-tenths of which is never used again); and they are encouraged to think in terms of career structures. Many are therefore deeply unwilling to accept that error is part of science and cannot be legislated away. This timidity is reminiscent of the widely held view that politicians should have blameless sex lives. The analogy appears in the question: "Otherwise, how can we trust them?" The minor answer (at least for scientists) is that it is seldom in their interests to lie: they are likely to be rumbled. The major answer is that trust should not be sought in science. It is a sign of the current intellectual malaise that it should be so widely yearned for. Scientists who need certainty should change their field to mathematics or logic. In a letter of 1817, John Keats wrote: "it struck me what quality went to form a Man of Achievement especially in Literature and which Shakespeare possessed so enormously — I mean Negative Capability that is when a man is capable of being in uncertainties, mysteries, doubts, without any irritable reaching after fact and reason". This applies to scientists of achievement too.

Error detection

I have little doubt that, if I chose, I could write and publish a paper plausibly claiming, say, that left-handed people have higher coital rates, on the average, than right-handers. The reader's job then is to assess the paper and decide what degree of belief to assign to the proposition rather than simply to refer to a list of dodgy authors. There is a whole array of skills that the reader should use to detect error. It is error that is important, not its cause; and error in science is far more common than outright fraud.

To concentrate on fraud is to overemphasize one (probably minor) cause of error. To detect error, the reader should ask whether I (the author of the hypothetical paper) am left-handed; whether I can reliably perform a Mann-Whitney test; whether I have devoted too much time to a hypothesis that is probably false; whether I am a fellow of All Souls or a student at the Tri-State College for Self-Improvement; whether left-handers are bigger liars than right-handers; whether I have used an unconventional definition of left-handedness; whether the coital rates were based on reported or recorded data; whether I am merely confirming the work of someone else (or making an original claim); and whether the *p*-value seems to be correctly assessed — and after all that, the reader should decide how much credence should be assigned to the paper. So why not add a query about fraud?

The scientific community should accommodate itself to the ineradicable presence of fraudsters. I am sceptical that fraud can be controlled, except by the prospect of being caught by subsequent researchers doing what Thomas Kuhn called "normal" science¹. The willingness to commit fraud seems widespread. A recent paper² reported that about a third of applicants for gastroenterology fellowships in the United States had misrepresented their academic accomplishments. The misrepresentation included citations of nonexistent articles in actual journals, articles in nonexistent journals and articles falsely identified as "in press". Among the recommendations of the authors were that "medical students and residents should be taught that embellishments of *curricula vitae* constitute misconduct". But such teaching would be redundant: those students knew that already.

Politics is often said to be about policies and not personalities. This is largely false. Similarly, it is wrong to think of science as a process independent of its practitioners. Scientists are people. Some are straightforward. Others are devious, confused, lazy, careless, psychotic, depressed, manic or the victims of domestic trouble.

Some scientists think of science as a game: we have to identify the rules by which they play. Some chessplayers are skilled in momentarily distracting their opponent's attention — and secretly rearranging the pieces. Some scientists are psychopaths: it is the business of other

scientists to detect signs of this condition. We should learn to associate some of these traits with certain authors — and assess their papers accordingly.

Sir Cyril Burt was the most famous psychologist in the world. It now seems that he may have fabricated data to support an hypothesis he believed. Lionel Penrose is credited with remarking of a lecture given by Burt: "I greatly admire the way the old boy says it — but I don't believe a word of what he says!" Penrose's robustness in the face of possible fraud deserves admiration. We should be looking not for scientists we can trust to tell the truth, but for scientists who get it right (a criterion Burt now seems to meet).

Risk motivation

Scientists seem to lack curiosity about what drives fraudsters. There clearly are several motivations, some of which are, admittedly, reprehensible. The more squalid fraudsters simply want tenure, or more research funds, or easy money from drug companies. They merit contempt. But others (for whom one may have some sympathy) go to immense trouble to pull an elaborate hoax. I doubt whether the harm done by scientific hoaxers is commensurate with the fuss made about them. Whoever planted the Piltdown skull provided an interesting problem for others to unravel, a stimulus for further research and some entertainment.

When we have been hoaxed, excessive censure is unseemly. We are the victims of a practical joke: our *amour propre* has been disturbed. But we should be no more Victorian about it than, say, about adultery. Scientific fraud should be regarded as perhaps regrettable, but certainly an ineradicable, interesting and integral part of science.

James Watson has proposed³ a swash-buckling set of rules for success in science, one of which is a preparedness to take risks. Let us think of scientific hoaxers as merely overstepping the limits of such preparedness. □

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Nature on the Internet (at last)!

From next week, *Nature* will maintain a site on the World Wide Web, but www.nature.com will not immediately contribute as radically to communication in research as will in due course be possible (but it may help people to find new jobs).

JUST as visitors to Japan feel naked without a good supply of calling-cards, so journals with a presence in science now seem desperately ill-provided if they do not also have a presence on the Internet. But that is only partly the spirit in which *Nature* will launch a site on the World Wide Web next week (19 October). There is every reason to believe that the information that we shall be providing from next Thursday will have immediate value for many readers, and that it will otherwise be instructive and even entertaining.

Yet it would be charitable if readers would regard what they find at <http://www.nature.com> as part of a learning process for *Nature*'s staff and for themselves. We are all in a strange state of uncertainty about the future role of the Internet. A facility for broadband communication between researchers must be of great importance to the international research enterprise, but it is too soon to know how it will be used most productively. *Nature* will learn a great deal from the months ahead; the central goal is to learn more about readers' needs.

Wishes are not always identical with needs. What *Nature* readers say they most need from *Nature* on the Internet is the facility to extract copies of research papers published in the current edition, and before the printed version appears at a nearby mail-drop after the usual series of stochastic happenings at the hands of the world's post offices.

At this stage, for reasons that are generally understood, we cannot provide that service. The technical difficulties of transmitting illustrations efficiently are well-known and much discussed. It is less well appreciated that even text containing letters of the Greek alphabet and the most common mathematical symbols becomes garbage if launched onto the Internet without sophisticated precautions against corruption. The software programs that make the handling of such texts manageable are cumbersome, and are not always installed on the machines of those who ask for access to these texts. There are also unsolved problems of security: how does a journal such as *Nature* ensure that only subscribers have access to its contents? Luckily, it will be a matter of months rather than years before those problems are solved.

Yet it is far from self-evident that full hard-copy versions of electronic texts are the research community's chief potential

benefit from broadband communications. Experience with other innovations in the technology of communications show that users adapt their habits to the new technology. Under the influence of the Internet, for example, the years ahead are likely to bring subtle innovations in the presentation of written texts to make their structure, and their connections with other texts, more readily apparent; then people may find themselves saving less and reading more widely, most obviously by means of hypertext links to documents listed in bibliographies. In short, it is unlikely that the the format of the standard scientific paper will survive the Internet intact, although it is too soon to guess what specific changes will come about.

But there is one opportunity that can be exploited here and now. It is the standard complaint of the authors of research that journals never give them enough room to say all they have to say. *Nature* is deemed particularly niggardly in this respect, although the facility for accompanying a research article with 'supplementary information' has been available for some years. Hitherto, copies of such material have been mailed free of charge to those who have asked for it. From now on, it will also be made available without restriction on www.nature.com.

There are the strongest reasons (apart from *Nature*'s strictly domestic need to be economical with its pages) why authors should seize this opportunity. There are many occasions when the availability of supplementary data will add force to the argument of a printed article. (Calibration measurements and control experiments are obvious illustrations.) There are also occasions when the detailed recitation of the technical details of an experimental procedure hampers understanding, or when the abbreviation of such an account

reaches the point at which an experiment cannot be replicated on the information given.

Nature does not intend to modify its present policy that published papers should be complete in themselves, but many authors will find the device of supplementary information a way of relieving present frustrations. *Nature* is however anxious that hard-worked referees should not be further burdened by having to wade through masses of data that are relevant but not central to a paper submitted for publication; in most circumstances, authors will submit this material on their own responsibility. (The accompanying box lists the formats for the time being acceptable.)

That is for the future. Next week and for the month ahead, www.nature.com will offer a somewhat miscellaneous collection of information. Perhaps of most immediate interest will be the weekly compilation of jobs advertised in the most newly current issue. There will also be information about *Nature*'s commercial interface with its subscribers (how to subscribe, or to complain about delivery, for example).

Readers will also find that www.nature.com includes a table of contents designed to be more suited to the VDU screen than is the printed version, that the most-current issue's news section is accessible (together with an archive reaching back for half a year) and that one of the current Opinion articles from that week's issue is presented in a form that (it is hoped) will stimulate responses from readers, which will themselves afterwards be accessible and which may even end up in print.

None of this prejudices what will appear on www.nature.com in future weeks and months. Like many others, the staff of *Nature* has launched itself on a learning curve, but does not yet know what it will eventually learn. Readers' comments and opinions will be essential to that process, but all concerned should remember that the printed journal remains central to our purpose, and must remain so at least so long as access to the Internet remains a luxury in many important parts of the world. Readers of ink-on-paper *Nature* need not fear that they will be assaulted by largely meaningless figures for the number of 'hits' scored on www.nature.com in any week. It will be time to boast when there is proof that the site has contributed to communication within the research community.

John Maddox

Supplementary information

Authors wishing to submit supplementary information for distribution through www.nature.com should use one of the following combinations of formats for text and graphic material:

For text in HTML (HyperText Mark-up Language), T_X and MS Word, use either GIF (Graphic Interchange Format) or JPEG (Joint Photographics Experts Group) for image files. Users of Postscript PDF format should know that their files will be transmitted for reading by Acrobat software.

Home for an orphan endorphin

David Julius

OPIATES have maintained their place in history as both the most valued and most abused of pharmacological agents. Drugs such as morphine and heroin are as unparalleled in their capacity to relieve pain and induce euphoria as they are in their ability to enslave the user in a cycle of tolerance and addiction. The two primary breakthroughs in understanding the molecular basis of opiate action were the identification of specific opioid receptors in the central nervous system, and the purification of endogenous morphine-like substances (endorphins) from the brain¹. On page 532 of this issue², Meunier *et al.* describe the

authors used this assay to follow ORL₁ agonist activity through a series of biochemical fractionation steps, yielding a heptadecapeptide with nanomolar potency at this site.

All five of the major opioid peptides characterized so far bear the amino-terminal signature sequence Tyr-Gly-Gly-Phe^{1,6}. Just as one might have hoped, the newly isolated peptide begins with the sequence Phe-Gly-Gly-Phe- and bears further resemblance to the opioid peptide dynorphin A. As is often the case for small peptide hormones and transmitters⁷, the novel peptide appears to be synthesized

that intraventricular injection of synthetic peptide into the mouse brain increases an animal's reactivity to pain, in curious contrast to the analgesic actions of most opiate drugs. In the absence of specific antagonists, the authors must rely on antisense technology to corroborate these findings; they show that a reduction in ORL₁ receptor expression has the predicted opposite effect of decreasing the animal's reactivity to noxious stimuli. Assuming that receptor levels are, indeed, reduced in mice treated with antisense oligonucleotides, these findings would support a role for the ORL₁ receptor and the novel peptide in a nociceptive pathway.

Of course, hyper-reactivity can reflect changes in systems other than those directly related to nociception. So before such a role for this peptide can be fully accepted, it will be necessary to define the sites of peptide biosynthesis and action within the pain modulation pathway using anatomical and physiological techniques such as radioligand binding assays, immunohistochemical and *in situ* hybridization methods, and stereotactic microinjection of agonists and antagonists into discrete regions of the brain and spinal cord. For now, it can at least be said that ORL₁ receptor transcripts are expressed in several areas of the central nervous system that are known to be involved in pain regulation, including the hypothalamus, brainstem and spinal cord dorsal horn^{4,5,8}.

Given the powerful euphoric and addictive properties of opiate drugs, it is important to consider the actions of opioid ligands in limbic system function and plasticity. ORL₁ receptor transcripts are, in fact, highly expressed in limbic system regions such as the cerebral cortex, amygdala and hippocampus^{4,5,8}. This may be interesting in light of findings that dynorphin can act as a neurotransmitter at glutamatergic mossy fibre synapses within the hippocampus⁹. By activating κ -opioid receptors in the mossy fibre pathway, dynorphin can depress synaptic transmission and inhibit the induction and expression of long-term potentiation. Nociceptin, acting at ORL₁ receptors, may play a similar role in modulating synaptic plasticity in the central nervous system. □

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Sequences of members of the endorphin family

Leu-enkephalin	Tyr-Gly-Gly-Phe-Leu-OH
Met-enkephalin	Tyr-Gly-Gly-Phe-Met-OH
β -Endorphin	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Val-His-Lys-Lys-Gly-Gln-OH
α -Neoendorphin	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys
Dynorphin	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-OH
Nociceptin	Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln-OH

isolation of a new seventeen-amino-acid-long peptide from rat brain that may be an endogenous agonist for an orphan member of the opioid receptor family.

Tentatively dubbed 'nociceptin' for its ability to induce hyper-reactivity in response to a noxious thermal stimulus, this peptide shares greatest sequence similarity with dynorphin A, one of five established endogenous ligands for opioid receptors (see table). These receptors have been classified into three main subtypes (μ , κ and δ) and cloning efforts have shown that they belong to a closely related subfamily of G-protein-coupled receptors³. Using homology-based screening strategies, several groups independently identified a novel member of the family that does not bind any of the known opiate ligands with high affinity (see ref. 2 and references therein). It was therefore proposed that the endogenous ligand for this orphan receptor (termed ORL₁ for opioid receptor-like 1)⁴ might be a novel opiate-related peptide⁵.

Working from the knowledge that all three opioid receptor subtypes are coupled negatively to adenylyl cyclase, Meunier and co-workers found that very high concentrations of the opiate etorphine can inhibit adenylyl cyclase in transfected cells expressing ORL₁ receptors⁴. Starting with crude rat brain extracts, the

as part of a larger polypeptide precursor. Thus, whenever such a precursor is found, there is the exciting possibility that additional peptide transmitters may be liberated by proteolytic cleavage at dibasic recognition sites that commonly flank the sequences of mature peptide hormones. If, as the authors suggest, this precursor polypeptide resembles pro-opiomelanocortin (from which β -endorphin and a variety of non-opioid peptides are derived), then they may have stumbled upon a treasure trove of new bioactive peptides.

A search of the gene bank database points out some additional bits of homology between the newly cloned precursor and other opioid genes within the presumptive pro-domain. This observation suggests that opioid ligands and their receptors may have evolved in parallel, maintaining some specific, high-affinity interactions along the way. Indeed, the novel peptide has no significant agonist activity at μ -, δ - or κ -opioid receptors⁵, and broad-spectrum opioid antagonists such as naloxone have no appreciable affinity for ORL₁ (ref. 4). How, then, should we view ORL₁ and its novel ligand — as functionally unique members of the opioid family, or as the first members of an entirely new peptide signalling system?

Meunier *et al.* present data to suggest

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The charge of the light brigade

Roger D. Blandford

It is now widely believed that, as well as accreting gas through a disk, the majority of quasars simultaneously expel gas with speeds as high as one-tenth the speed of light. However, the origin and dynamics of this gas remain a mystery. This month, writing in the *Astrophysical Journal*, Murray *et al.*¹ have proposed a boldly original theory of gas flow in quasars that challenges some of the standard assumptions made in modelling quasars and is likely to provoke a strong observational and theoretical response.

By the latest count, there seem to be over thirty billion galaxies in the Universe. Of these, a good fraction develop a pathology at some stage in their lives. A very small nucleus becomes prodigiously luminous and outshines all the stars that surround it so that what we see itself looks like a star, albeit one that is over a hundred billion times as powerful as our own Sun. Because of this, we call these active galaxies quasi-stellar objects — quasars for short — and at any time there appear to be over three million of them visible from the Earth².

Not surprisingly, astronomers have been enticed into scrutinizing the nuclei of galaxies in fine detail and have, over many years of painstaking observation and theoretical contemplation, pieced together a physical model for how they work³. It is thought that the prime mover for this tremendous activity is a black hole more massive than a billion suns, which attracts gas discarded by surrounding stars and small satellite galaxies into its voracious maw. This gas, which begins its journey far away from the black hole, is likely to have some circulatory motion and as it sinks downwards, the importance of this circulation increases so that the gas eventually settles into an orbiting ring or disk.

Frictional forces, probably attributable to the influence of unstable magnetic fields, drive the gas inwards, all the while releasing some of its gravitational potential energy in the form of radiant energy. As the gas approaches the black hole, an appreciable fraction, perhaps ten per cent, of its total rest mass (mc^2) can be liberated in this manner. This exceeds by a factor of roughly a hundred the energy yield from atomic nuclear reactions and is consistent with the large power of quasars.

In this standard (though still somewhat conjectural) model, the black holes themselves are no larger than our Solar System (10^{15} cm) and most of the primary energy release occurs within a surrounding sphere of radius 10^{16} cm, tiny relative to the radius of the galaxy itself (10^{23} cm). The radiation originates from such a small area that much of it emerges as roughly

black-body emission with frequencies in the ultraviolet region of the electromagnetic spectrum. This ultraviolet continuum emission explains another prominent feature of quasars, their broad emission lines. What is generally assumed is that a small quantity of gas in the form of clouds is illuminated at radii of 10^{18} – 10^{19} cm by the central ultraviolet continuum source and this gas reprocesses the incident radiation as ultraviolet resonance lines of the most common ions, such as C^{3+} , N^{4+} and so on. These emission lines are observed to be broad, a feature that is conventionally associated with cloud velocities as large as a few per cent of the speed of light. Modern photoionization codes boast considerable success in reproducing the measured line ratios.

Now, roughly ten per cent of all quasars also exhibit broad 'absorption' lines corresponding to similar transitions to the prominent emission lines but blue-shifted with respect to them⁴. This is associated with gas that streams radially outwards with speeds as large as $0.1c$. Where it is directed towards us, in front of both the continuum source and the emission line regions, it scatters (without actually absorbing) the continuum photons out of the line of sight. Moderately strong arguments⁵ suggest that most quasars expel gas in an equatorial fan covering about one steradian and that we only see the broad absorption lines when our line of sight passes through this fan.

It is a seemingly inevitable consequence of the standard photoionization models that the scattering gas be located beyond 10^{18} cm and, like the denser, emission line gas, it takes the form of a spray of tiny clouds, in this case as small as 10^8 cm. This has then raised the question 'What agency accelerates and confines these clouds and prevents them from quickly disintegrating and dispersing?' The usual suspects, radiation, magnetic field, rotation and cosmic rays have all been paraded in the literature^{6–8}, but we still lack a conviction. It appears that getting the atomic physics right first and then trying to accommodate the dynamics has led to scientific stasis.

What Murray *et al.*¹ have done is first to invert this procedure and ask 'What is the most natural way to accelerate the gas to the high speeds observed?' and then to try to fix up the photoionization modelling.

To accelerate the gas, they appeal to the oldest and most natural suggestion, namely that the continuum photons that are scattered out of the beam also pass on their radial momentum to the gas. Absorption goes hand in hand with acceleration⁹. For this, there is some observational encouragement as there is now

spectroscopic evidence for radiative acceleration in the detection of features in the absorption spectrum of a prominent line of C IV that correspond to the wavelength separation of Lyman- α and a nearby line of N V. This has been found in the few broad absorption line quasars where it is expected¹⁰.

The radical element of their model is that they propose that the gas is not a tenuous spray of clouds but a continuous wind originating from the surface of the disk of accreting gas. Dynamically, this makes sense and they are able to reproduce the observed line profiles. However, they are forced to conclude that the gas is accelerated at radii 10^{16} – 10^{17} cm where the intensity of the radiation is likely to be so large as to preclude the survival of the ions that are directly observed. To rectify this, they then add a low-pass filter of dense 'hitch-hiking' gas appended to the inner surface of the outflow with provenance as obscure as its purpose. This filter has properties that are carefully tuned to transmit the ultraviolet radiation that provides the acceleration and to block the soft X-rays that would otherwise over-ionize the gas. This is the opposite of what is usually presumed. It remains to be seen how naturally this shield can be formed and how robust are its properties. As a bonus, though, the authors argue that the emission lines are formed in the innermost parts of the flow, again at much smaller radii than is conventional.

Commendably, this model makes several testable predictions. The size and kinematics of the emission line region can be probed using the technique of reverberation mapping, hitherto only applied to Seyfert galaxies. More high-ionization species such as Ne VIII should be present than are usually reported. Absorbing quasars should not be soft X-ray sources and radio-loud quasars ought to show more evidence for absorption lines than they have thus far. There should be strong continuum polarization caused by electron scattering and the lines themselves may be intrinsically variable.

These forecasts are made at a propitious time when the observational capability to test them is becoming increasingly available, and we should soon know how

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well they are borne out by experience. If they are confirmed, then we will have taken one step closer to the central black hole that we believe lies at the heart of every quasar. Even if the disk-wind model for line formation perishes, it will do so nobly as it will have rightly forced photoionization modellers to pay far more

attention to the dynamical state of their gas clouds than they have been wont to do in the past. □

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EVOLUTIONARY BIOLOGY

A twofold tragedy unfolds

Peter Hammerstein

ADAM Smith considered that competition between businessmen tends to promote the well-being of society, but when it is unchecked competition can have quite the opposite effect. It is not just humans that need to keep competition under control, and on page 520 of this issue¹ Frank proposes a model to explain how genetic evolution can exert such restraining mechanisms.

When too many individuals use renewable resources that are scarce but freely available, the unnecessary degradation of these resources is typically a consequence². For example, communal pastures may be overgrazed as a result of a dairy farmer bringing in extra cattle in order to produce more milk for himself, even though his behaviour reduces the total milk production on the pasture, forcing other farmers to share the cost of his behaviour.

Many human communities have reacted to this 'tragedy of the commons' by introducing institutions that limit access to public resources³. For example, an alpine village in Switzerland imposed the so-called 'winter rule' by which no citizen was allowed to send more cows to the public meadows than he could feed during the winter⁴. This rule was enforced by inspection and substantial fines. The citizens had to pay a price for maintaining this policing mechanism which enhanced their overall economic efficiency. A look to biology also turns up examples of policing, and Frank's general selection model¹ addresses the question of how genetic evolution can generate this costly control and thereby resolve the tragedy of the commons.

What examples do we have of biological policing? Perhaps the most intriguing case of regulated competition is in the intra-organismic process of meiosis, during which two homologous chromosomes are both given a fair chance of entering vehicles (gametes) into the next generation. Furthermore, chromosomes themselves probably evolved from unlinked genes as a means to escape the tragedy of the commons within protocells. Chromosomes keep mutually cooperative genes together by genetic linkage. This reduces a cooperative gene's risk of being exploited by others that replicate particularly fast at the expense of overall cell performance.

Chromosomes are an expensive means of defeating the tragedy of the commons as their replication is slow compared with isolated genes⁵.

At the organismic level, social insects are particularly promising subjects for the study of policing mechanisms. Despite the hierarchy implicit in the terms 'queen' and 'workers', the workers themselves seem to exert most of the control over large colonies⁶, including the active prevention of egg-laying by others.

Rather than dealing with an example in detail, Frank makes an attempt to discuss some general conditions under which policing mechanisms can evolve. In his model he looks at an evolving population in which individuals (or smaller units) interact in groups. Within a group there is a certain degree of genetic relatedness. Individuals can behave very competitively or exert some degree of self-restraint. Their reproductive success relative to other group members depends on their competitiveness. There is also a selection effect at a higher level in that intra-group competition decreases group performance. The second strategic parameter is an individual's contribution to policing. The policing mechanism as such is not made explicit in the model. As the group's total contribution to policing increases, policing progressively reduces the negative effect of intra-group competition.

Frank emphasizes the following conclusion from his analysis. A high degree of relatedness between group members counteracts the evolution of policing. The essential reason is that self-restraint based on the logic of kin selection can achieve for free what expensive policing would bring about. Now, if one looks at sufficiently low degrees of relatedness, this argument breaks down. Contributions will then be made to the policing mechanism provided it is cheap enough. Frank finds it surprising how strongly natural selection favours these contributions in his model. He also maintains that there will be a double moral standard: pay for policing but strive very competitively for your own success in the group.

If it can evolve so easily, why is policing not found more often in nature, and why

are those evolutionary steps fairly exceptional that created higher-level units, such as superorganisms, by suppressing lower-level competition⁷? To deal with these questions, it pays to have a closer look at Frank's model. In my opinion, one aspect of his mathematical results needs particular attention, although it is not emphasized in his paper. Frank's policing equilibrium only exists if a rather stringent condition is satisfied. This condition requires the individual cost of policing to be kept within a limit that depends on genetic relatedness. This limit, however, converges to zero as we look at ever smaller degrees of relatedness. In other words, policing has to be vanishingly cheap in order to evolve in groups of fairly unrelated individuals. But we would hardly expect an almost cost-free mechanism of policing to exist. This very strongly confines the scope for the evolution of policing in Frank's model.

To put this in a wider context: when we look at examples of the tragedy of the commons, we usually find another, similar tragedy associated with it. The refusal to pay for policing has a substantial positive effect on the individual who does not contribute (strong feedback), but the reduced efficiency of the policing mechanism is shared with the competitors (low feedback). This creates an incentive not to pick up the tab, so the tragedy of the commons is twofold. In biological evolution the second tragedy makes it very difficult to overcome the first.

From Frank's paper we learn that the second tragedy becomes a little less dramatic when there is genetic relatedness. Intuitively, this can be seen if one thinks of the problem in terms of personal fitness. As strategies of relatives are correlated, an individual's payment for policing gets multiplied by the correlated payments of its relatives. Intermediate levels of relatedness therefore facilitate the evolution of policing. This is the major take-home message of Frank's paper. But it does not follow from his study that policing should evolve under many circumstances. The escape route of the tragedy of the commons is too easily blocked by the associated cost tragedy. This will only become transparent if we develop models with explicit mechanisms of policing. □

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Egg bank investment

Winfried Lampert

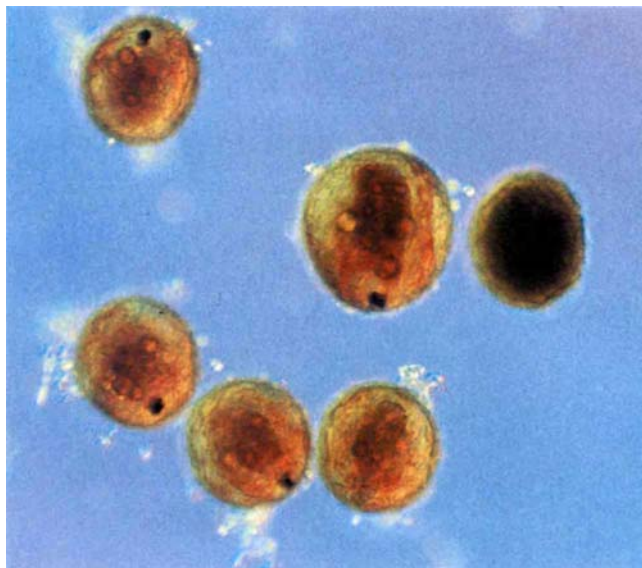
PLANKTONIC organisms in lakes are usually small and short-lived, responding quickly to changes in the environment and showing typical seasonal successions¹. But some manage cunningly to increase their lifespan by assuming a dormant stage that settles into the sediment, where they wait for favourable conditions to establish new populations. These resting stages can survive impressive periods of diapause: for example, akinetes of the cyanobacterium *Anabaena* have survived for 60 years², and zooplankton eggs as old as 35 years are still viable. But now, writing in *Ecology*³, Hairston and his co-workers report a new world record: from the sediment of a small freshwater lake in Rhode Island, they have hatched resting eggs of the copepod *Diaptomus sanguineus* that are probably 330 years old.

Diaptomus sanguineus is a small (1 mm body length) calanoid copepod, distributed throughout most of North America, that lives in permanent lakes and ponds as well as in temporary ponds. In winter it produces eggs that hatch quickly and develop immediately into nauplius larvae, but it switches to diapausing resting eggs in spring to escape drying out in temporary ponds during the summer and to avoid predation by planktivorous fish in permanent water bodies⁴. The diapausing eggs are about 100 µm in diameter. They possess a thick chitinous shell that makes them resistant to desiccation and allows them to survive passage through a predator's gut.

Hairston *et al.* selected two small lakes for their study. The larger one is permanent, with a surface area of 2.4 hectares and a maximum depth of 4.0 metres. The smaller one (0.15 hectares, 1.5 metres) dries out occasionally — the last time was in 1981. Scuba divers took sediment cores which were sliced into 1-cm layers. *Diaptomus* resting eggs were isolated from each layer, then counted and hatched in an incubator. Sediment slices from a parallel core were dated by ²¹⁰Pb activity. This allowed the calculation of sedimentation rates (0.5–0.8 mm per year) and indirect dating of the eggs found at different sediment depths.

Although egg numbers declined with sediment depth (mortality rate was about 1.5 per cent per year), some eggs were still present 31 cm below the surface in the larger lake and were estimated to be

about 400 years old. The mean egg age in the lake was about 70 years. As dead eggs decay quickly, it was fair to assume that these eggs were still viable, although the oldest eggs that could be hatched were 330 years old. Only a fraction of the isolated eggs could be hatched from all depths, but this is not unusual as the optimum environmental cue to terminate diapause and induce development of the embryo is



Six diapausing eggs of *Diaptomus sanguineus*. Five of the eggs are in the 'eyed-embryo' stage into which the eggs develop after they are removed from the lake sediment. The sixth egg (right) is in an earlier developmental stage typical of eggs buried in the sediments. The mean egg diameter is about 100 µm. (Photograph by C. M. Kearns taken using phase-contrast illumination and blue filter.)

still unknown (probably a combination of oxygen, light and temperature).

What is the significance of this remarkable result? Diapausing egg densities at the sediment surface were about $5 \times 10^4 \text{ m}^{-2}$ and the total number of eggs waiting to hatch in the larger of the two lakes was about 6.5×10^9 , which is 18 times greater than the annual mean maximum population size of adult copepods in the open water. These eggs represent an egg 'bank', analogous to the seed banks of many terrestrial plants. Simultaneous hatching of larvae from eggs produced in different years produces a large overlap between generations, which slows the rate of microevolution⁴. It also decreases the rate of elimination of a species from a system when conditions for active stages become unfavourable, and increases the rate of recolonization of a water body when conditions improve. With non-overlapping generations, a single harsh year with failure of reproduction will result in the elimination of the species from the lake or even a given region. The system may be

slowly recolonized by dispersal of organisms from unaffected lakes. The egg bank functions as a time-dispersal system that allows immediate recolonization.

This idea has been further developed with respect to the possible consequences of global climate change on lake biota⁵. A change in mean temperature could well be accompanied by a change in its variance⁶. Zooplankton that cannot physiologically tolerate a series of extreme years can still survive by investing in an egg bank. Hence the egg bank may become increasingly important for species diversity and the structure of lake foodwebs, which has an indirect effect on lake productivity and nutrient dynamics.

Seed-bank theory predicts that fluctuations in recruitment should lead to the evolution of prolonged diapause⁷. There is not enough known yet about egg banks to decide whether they are adaptive or just an artefact. Will the eggs buried in the sediment ever hatch in nature? Hairston *et al.* think that this is possible if the sediment is perturbed and old eggs are moved to the surface where they experience the proper environmental cues. There are many possibilities for sediment disturbance from feeding fish to falling trees to limnologists' anchors.

One striking example of how sediment disturbance can affect lake plankton communities was reported when the top sediment layers of Lake Trummen in Sweden were removed for restoration purposes⁸. This mechanical disturbance was followed by the appearance of large maxima of chrysophycean algae in the lake, probably from cysts that were liberated from lower sediment layers. The reminder of such findings, and Hairston and colleagues' dramatic discovery of survivors of environmental conditions from several hundred years ago, will stimulate numerous new studies in ecology and population genetics. □

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Clamping the TBP stirrup

Steven Hahn

INITIATION of messenger RNA transcription by the enzyme RNA polymerase II involves a complex protein machine, which is responsible for promoter recognition and response to regulatory signals. A major advance in understanding the workings of this cellular machinery has been accomplished with the publication of two papers, one in *Cell*¹, the other in *Nature*², describing the nuclear magnetic resonance and crystal structures of transcription factor TFIIB.

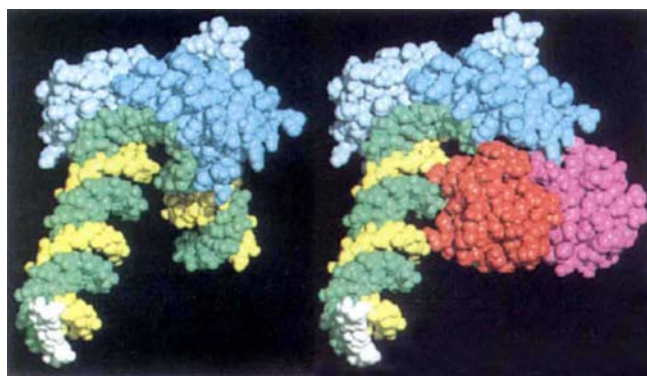
TFIIB binds to the TATA-binding protein (TBP)-DNA complex, where it in turn recruits and positions a complex of RNA polymerase II (pol II) and TFIIF. TFIIB has been conserved from Archaea to humans, and a TFIIB homologue (BRF1) assists initiation by RNA polymerase III, which catalyses synthesis of transfer RNA (reviewed in ref. 3). Intense interest has centred on TFIIB, as it has been implicated as an important target of transcriptional activators⁴. The new structures answer some long-standing questions about TFIIB and its interaction with the preinitiation complex, and provide the basis for future studies on the workings of the transcription machinery.

TFIIB consists of an amino-terminal region, with a putative metal-binding site, connected to a protease-resistant carboxy-terminal core (IIBc) composed of two long amino-acid repeats. The carboxy-terminal core can bind to the TBP-DNA complex, but fails to recruit a complex of pol II-TFIIF (reviewed in ref. 5). Both groups have solved the structure of the TFIIB core domain, one of IIBc in solution using NMR¹, the other of a complex between TBP, IIBc and a TATA-box-containing promoter². IIBc contains two similar domains (each encoded by one of the two repeats) connected by a short, random-coil peptide. Each of these repeats consists of five α -helices arranged in a compact globular domain. In striking agreement with a prediction based on sequence alignment between TFIIBs and cyclins⁶, the structure of each repeat in TFIIB can be superimposed on the corresponding repeat of cyclin A (ref. 7).

One unexpected finding upon comparison of the NMR and crystal structures is a major conformational change between the structure of TFIIB in the TBP-IIBc-DNA complex and of IIBc in solution. Orientation of the two repeats differs by about a 90° rotation and a 30° twist, and the first

helix of the second repeat is about 1.5 turns longer in the DNA-bound form, with a corresponding shortening of the linker separating the two domains; this apparent conformational change upon DNA binding will have to be confirmed, but it could indicate a rate-limiting and potential regulatory step in the initiation process.

In the crystal structure, IIBc makes a complex series of interactions with both TBP and DNA. TFIIB binds underneath and on one face of the TBP-DNA complex, where it clamps the carboxy-terminal



Space-filling models of complexes between the TATA-binding protein (TBP) and DNA. Left, the TBP-DNA complex. Right, the TBP-IIBc-DNA complex. The TBP N-terminal repeat is light blue and the C-terminal repeat dark blue; the IIBc N-terminal repeat is red and the C-terminal repeat magenta. (Figure by D. Nikolov and S. Burley.)

stirrup of TBP and contacts DNA both upstream and downstream of the TATA box. Surprisingly, all contacts between IIBc and DNA involve interactions with the sugar-phosphate DNA backbone; no interactions are seen in the major or minor grooves of the DNA. This implies that TFIIB binding will be essentially independent of sequence.

The positions of DNA contact agree precisely with those determined by hydroxyl-radical footprinting⁸, and also with the fact that TFIIB crosslinks to DNA both upstream and downstream of the TATA box (T. Lagrange *et al.*, personal communication). DNA contacts are made by an astounding total of four α -helices and one interhelix loop. Most of the contacts are made by the amino-terminal TFIIB repeat. Owing to the unusual structure of DNA in the complex, several TFIIB residues make complex interactions with the DNA. For example, the arginine residue at position 193 contacts a phosphate at position -30 on the top strand as well as phosphates -23 and -22 on the bottom strand of the promoter. TFIIB also makes extensive contacts with TBP, most of which (9/13) are between the amino-terminal repeat of

IIBc and the carboxy-terminal stirrup of TBP. This agrees exactly with the results of TBP mutagenesis⁹ as well as the position of DNA contacts measured by hydroxyl-radical footprinting⁸.

TFIIB binding is asymmetric, with binding on one face of the TBP-DNA complex and non-equivalent interactions being made between the two TFIIB repeats and TBP-DNA. The asymmetric interactions with TBP-DNA help explain why transcription occurs in only one direction (downstream of the TATA box). Although the amino-terminal region of TFIIB was not crystallized, it is predicted to be close to the start site of transcription. So it would be in the right position to interact with pol II and TFIIF (ref. 10).

TBP binding to DNA induces a remarkable deformation in the structure of TATA-box DNA (refs 11, 12). Strikingly, TFIIB binds the preformed TBP-DNA complex without causing any further conformational change in either TBP or DNA. This is partly because TFIIB interacts with only the DNA backbone and does not require access to functional groups inside the major or minor grooves. Mutagenesis and DNA-deletion studies have shown that both TBP and DNA are an essential part of the target for TFIIB binding^{8,9}, indicating that the target for TFIIB is in fact TBP bound to distorted DNA. In the absence of DNA distortion by TBP, TFIIB could not interact with DNA both upstream and downstream of the TATA box.

Finally, the TFIIB structure raises questions about the role of TFIIB in activation. A mutation in yeast TBP in the carboxy-terminal stirrup⁹, in which a leucine residue at position 189 is substituted by a lysine, blocks TFIIB interaction with TBP-DNA and abolishes the ability of VP16 to stimulate transcription without affecting basal transcription. In the crystal structure, the leucine at position 189 of TBP makes a direct contact with TFIIB. Rather than inducing a conformational change in TBP or TFIIB (or both), as proposed, it may be that this mutation simply

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imposes a new rate-limiting step on the initiation process by slowing the binding of TFIIB or destabilizing the TBP-TFIIB-DNA complex. This slowed step may have no apparent effect on basal transcription, because it is an inherently inefficient and/or slow process. But it may have a profound effect on multiple rounds of activated transcription which must pro-

ceed rapidly. However that may be, the new structural information will lead to many more insights into the mechanism and regulation of gene expression. □

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SYNTHETIC CHEMISTRY

Mimicry with gold

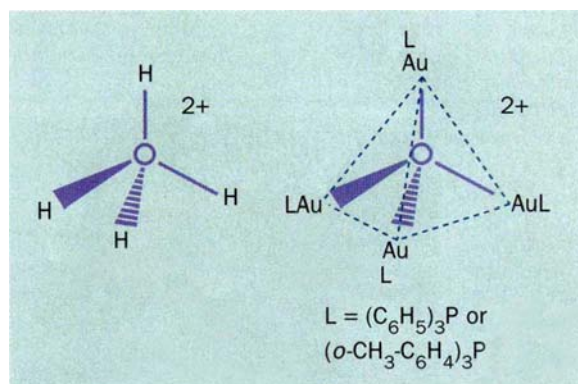
G. K. Surya Prakash

PROTONATED water (hydronium ion, H_3O^+), an important species involved in the Brønsted acid-catalysed reactions of aqueous solutions, has been well characterized¹. G. Olah has suggested² that diprotonated water, H_4O^{2+} , might be formed as an intermediate in superacid media by further protonation of H_3O^+ . Using a gold (I) organometallic fragment (LAu^+) as an isolobal substitute for H^+ (two fragments are isolobal if their symmetry properties, approximate energy, shape of frontier orbitals and number of electrons are similar)³, Schmidbaur *et al.* have now prepared doubly charged tetrahedral gold complexes⁴ that are excellent analogues of H_4O^{2+} (page 503 of this issue). This structural characterization of an isolobal analogue of diprotonated water is fascinating in itself, and gives further credence to the suggested involvement of H_4O^{2+} in superacid media.

H_4O^{2+} is an important member of the class of main-group (first row) gitonics (a gitonics cation is one where the two positive charges are formally located on the same atom)². Indirect evidence for the transient existence of H_4O^{2+} -like species comes from observations of hydrogen-deuterium exchange of H_3O^+ in highly acidic deuterated superacids such as $\text{DF}:\text{SbF}_5$; the exchange rate increases with increasing acid strength⁵. Although *ab initio* calculations indicated that H_4O^{2+} prefers to deprotonate, a tetrahedral symmetry structure (as shown in the figure) was found to be the global minimum and computed to have considerable kinetic stability, with a barrier of 38.2 kcal mol⁻¹ for deprotonation⁶. The methylating ability of the cation (CH_3O^+ , an analogue of H_3O^+ , is enhanced in strong Brønsted acid solutions, indicating further activation of the lone pair on oxygen: the assumption has been that the analogous dication ($\text{CH}_3\text{O}^+\text{H}^+$) is a methylating species⁷. Likewise, the reactivity of NO_2^+ and CH_3CO^+ also increases in strong acid solutions; here, gitonics NO_2H^+ and CH_3COH^+ dications are thought to be the reactive species involved in electrophilic nitrations and acetylations of

deactivated aromatic compounds and hydrocarbons⁸. Such activation of electrophiles in strongly acidic Brønsted acids is known generically as protosolvation⁹. But these reactive species, if they exist, are too unstable to be isolated and observed directly — hence the interest in their more stable analogues.

The approach of constructing analogues for fleetingly existent species is not entirely new. Isolobal analogies are useful



Doubly protonated water, H_4O^{2+} , and its gold cluster analogue as prepared by Schmidbaur *et al.*⁴.

for understanding the underlying bonding principles of diverse chemical systems. Schmidbaur's group has previously succeeded in making several other intriguing structures relevant to higher-coordinate main-group element chemistry. One such is $[(\text{C}_6\text{H}_5)_3\text{PAu}]_3\text{C}^+$, an analogue of CH_5^+ (ref. 6). The CH_5^+ ion, which has five ligands around a 'supervalent' carbon, has been characterized by mass spectrometry. The importance of CH_5^+ as an intermediate in the oligocondensation of methane to higher hydrocarbons under superacid catalysis was demonstrated by Olah¹ in the late 1960s. The CH_5^+ cation is considered the prototype of nonclassical carbocations, as it contains a five-coordinated central carbon atom (also called hypercarbon) with four valence electrons (note that even with five ligands the octet rule of carbon, a first-row element, is not violated¹). The trivalent tricoordinate CH_3^+ cation, on the other hand,

is considered the parent of classical carbocations¹. For CH_5^+ , high-level theory predicts a structure with C_s symmetry⁷. Schmidbaur's analogue, however, had a C_3 symmetry structure in the crystal lattice. The discrepancy is probably due to substantial Au-Au bonding in the analogue.

Other intriguing structural mimics^{8,9} are octahedral $[(\text{C}_6\text{H}_5)_3\text{PAu}]_6\text{C}^{2+}$, an isolobal analogue of diprotonated methane² (so far not found experimentally); CH_6^{2+} (the structure CLi_6^{2+} has been probed by *ab initio* theory⁸); and $[(\text{C}_6\text{H}_5)_3\text{PAu}]_5\text{N}^{2+}$, an analogue of as yet hypothetical NH_5^{2+} . Similar analogies exist between carbocations and isoelectronic and isostructural carboranes and boranes¹.

The relevance of the polyhedral gold clusters to carbon chemistry is also indicated by the crystal structure of the empty $[(t\text{-Bu})_3\text{PAu}]_4^{2+}$ cluster (that is, a cage without a central atom) wherein substantial metal-metal bonding exists in a tetrahedral geometry¹⁰ resembling the tetrakis-*t*-butyltetrahedrane, $(t\text{-Bu})_4\text{C}_4$ (ref. 11). In principle, the addition of a neutral oxygen atom to such an empty cluster should lead to new H_4O^{2+} analogues. As pointed out by Schmidbaur *et al.*⁴, because gold does not form a stable binary oxide phase, subsurface oxides with unique bonding patterns like those observed in the cluster complex that they have been studying may play a unique role in catalysis of gold and related metal surfaces.

Perhaps the most important implication of the work by Schmidbaur *et al.* is how well the structures of positively charged gold cluster complexes relate to bonding motifs in the main-group electron-deficient ions of carbon, nitrogen and oxygen in superacid media. This is true despite the fact that, in gold cluster complexes, Au-Au bonding

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holds the ligands in place around the central atom, rendering the complexes stable (this is termed 'aurophilicity' by Schmidbaur *et al.*). Although many of the gtonic main group dications may never be observed as long-lived, persistent species in superacid media, the characterization of these isolobal gold analogues gives

additional support for their transient existence and involvement in many superacid-catalysed processes. □

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FLOWER DEVELOPMENT

LEAFY blooms in aspen

George Coupland

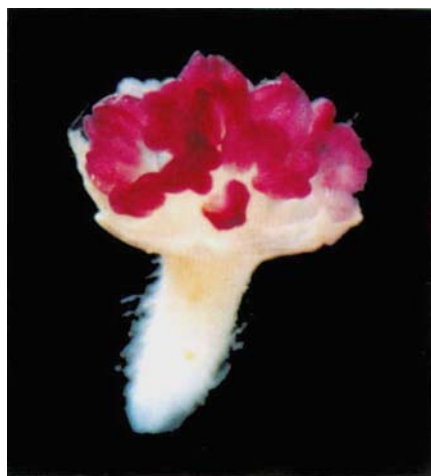
THE expression of a single gene, under the control of a viral promoter that is active in all *Arabidopsis* cell types, is enough to trigger the development of flowers from groups of undifferentiated cells. This discovery is reported in two papers in this issue, one by Weigel and Nilsson on page 495¹ and the other by Mandel and Yanosky on page 522². The flowering switch is either *LEAFY* (*LFY*) or *APETALA1* (*API*), two genes previously identified by homeotic mutations in *Arabidopsis* that partially transform flowers into shoots³. Moreover, the function of *LFY* is likely to be highly conserved in unrelated plant species, for expression of the *Arabidopsis* gene from the same viral promoter results in a similar phenotype in transgenic aspen trees⁴.

The new results demonstrate for the first time that expression of single genes is sufficient to confer floral identity on primordia that would form other organs in wild-type plants. This discovery followed the observation that transcripts from both *LFY* and *API* are present only in floral primordia and later floral cell types³, but the role of single genes has been difficult to assess because *LFY* and *API*, as well as at least two other genes, have overlapping functions^{3,4}.

Leaves, flowers and inflorescences of *Arabidopsis* are derived from the shoot meristem, which is composed of undifferentiated cells, often compared to animal stem cells, located at the apex of the shoot. Each of these organs develops from primordia that appear as outgrowths of cells in defined positions on the flanks of the shoot meristem. The organs develop in the characteristic order of leaves, followed by inflorescences and then flowers. Leaves are formed during vegetative growth, and inflorescences and flowers during reproductive development.

Several genes, including *API*, *LFY*, *APETALA2* and *CAULIFLOWER*, are required in the early stages of flower development to ensure that floral primordia form flowers rather than inflorescences³. These genes, all of which probably encode transcription factors, are often described as floral meristem-identity genes. They have overlapping functions,

because mutations in single genes cause incomplete transformation from flowers to inflorescences, but plants carrying mutations in more than one of them virtually have their flower development abolished⁴. The new results^{1,2} demonstrate that ectopic expression of either of two of these genes alters the morphology of the plant in several ways: the transition from vegetative growth to reproductive development occurs earlier so that fewer leaves are formed; single flowers are formed in place of inflorescences; and the shoot meristem is finally consumed in the development of a flower that terminates shoot growth.



Flower power — a male flower formed in a leaf axil of a 7-month-old transgenic hybrid aspen constitutively expressing the *LEAFY* gene from *Arabidopsis*. Aspen species normally flower only when they are at least eight years old. (Photo by Ove Nilsson.)

Termination of the shoot with a flower is also a feature of terminal flower (*tfl*) mutants of *Arabidopsis*, and the *LFY* gene is expressed ectopically in the shoot meristem of *tfl* mutants^{5,6}. The characteristic indeterminate morphology of *Arabidopsis* plants, which continue to form flowers on the flanks of the meristem for an indefinite period, is therefore probably a consequence of TFL repressing the expression of floral-meristem identity genes in the shoot meristem, and of the floral-meristem identity genes promoting floral devel-

opment on the flanks of the meristem.

Variations in the balance of *TFL* and *LFY* function can explain some of the diversity in shoot morphology found in different plant species⁷. The pattern of *TFL* transcription in the transgenic plants ectopically expressing the floral meristem-identity genes cannot be tested because the *TFL* gene has not yet been cloned. So the terminal flower of the transgenic plants carrying the fusion of the viral promoter to the *LFY* and *API* genes could be a consequence of *LFY* and *API* repressing *TFL* expression at the shoot meristem, or of their being able to promote floral development even in the presence of TFL.

The relationship between ectopic expression of the floral meristem-identity genes and the transition from vegetative to reproductive development is less clear. Ectopic expression of *LFY* or *API* is apparently not sufficient to direct floral development immediately after germination, but a period of vegetative growth occurs and, as in wild-type plants, its duration is sensitive to environmental conditions such as photoperiod.

The requirement for vegetative growth before *LFY* or *API* can cause floral development indicates that the shoot meristem and primordia of very young plants cannot respond to these genes. *Arabidopsis* genes required to promote the transition from vegetative to reproductive development were recently isolated^{8,9}, and others have been identified by mutation. In wild-type plants, the flowering-time genes act before *LFY* and *API*, and there is genetic evidence for interactions between mutations affecting flowering time and *lfy* (ref. 9). If some of the flowering-time genes are not active in early vegetative development, and if their products must be present before the floral meristem-identity genes can act, then this might explain why ectopic expression of *LFY* or *API* does not lead to flowering almost immediately after germination.

The precocious flowering of transgenic plants ectopically expressing the floral meristem-identity genes suggests a potential for application in biotechnology. In agriculture, for example, optimization of flowering time to the geographical location in which the crop is grown can be a key determinant of yield, particularly when the crop is grown at the extremes of its range. However, the morphological abnormalities shown by the transgenic plants might outweigh any advantage caused by improved flowering time. Altered flowering time of crop plants is more likely to be achieved by modifying the expression of flowering-time genes, at least one of which also causes precocious flowering when its transcript becomes more abundant⁹. So far, however, it is unclear whether these flowering-time genes can be used in species unrelated to the plant from which they were isolated. ▶

The observation that the *Arabidopsis* *LFY* gene can cause transgenic aspen plants to flower many years earlier than they normally would, illustrates how conserved the function of the floral meristem-identity genes is. Moreover, it suggests that modification of their expression might be useful in non-agricultural applications, such as modifying horticultural

species to create novel plant morphologies or to regulate their flowering time, or in promoting the flowering of trees so that they can be rapidly cross-pollinated to produce new varieties. □

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protein kinase A mutants, these motor proteins still co-localize with *oskar* mRNA, but this time to the centre of the oocyte^{8,10}.

Given all this evidence for microtubule-based transport, how can the requirement in *oskar* mRNA localization for tropomyosin and, by implication, the actin cytoskeleton be explained? One possibility, which can probably be discounted, is that tropomyosin mutants block *oskar* mRNA transport by altering the microtubule organization, as both dynein and kinesin- β -galactosidase localize normally in mutant oocytes³.

An attractive alternative is that an actin-based transport system at the anterior of the oocyte delivers *oskar* mRNA to the microtubules for transport to the posterior. This model of local traffic along actin and long-distance transport along microtubules is consistent with the tropomyosin-mutant phenotype — most *oskar* mRNA could get trapped at the anterior because it cannot be transported along F-actin or because the actin is misorganized, but the small amount that does reach the microtubules can be transported all the way to the posterior pole. There is also a precedent for transport particles that can move along both actin and microtubules: single vesicles can switch between the two motility systems in extruded squid axoplasm¹¹.

There are still many questions that need answering before the role of tropomyosin in *oskar* mRNA transport can be resolved. For example, it would be nice to know the distribution of tropomyosin in the oocyte, and the arrangement of actin at the anterior pole. Nevertheless, the new results show that *oskar* mRNA localization is surprisingly complicated, and that it involves both the actin and microtubule cytoskeletons. In future, the *Drosophila* oocyte should provide a powerful model system in which the tricks of developmental genetics can be used to address basic questions in cell biology. □

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DEVELOPMENTAL BIOLOGY

New role for tropomyosin

Daniel St Johnston

THE interests of cell biologists and developmental biologists often pursue separate paths, with the former tracing the goings-on inside a single cell while the latter follow the interactions between cells that lead to complex patterns. One area where these paths look like converging is in anterior-posterior axis formation in *Drosophila*, in which the polarity of the whole organism is defined by the localization of two messenger RNAs, *bicoid* and *oskar*, to opposite ends of a single cell^{1,2}. The mislocalization of either mRNA causes pattern defects in the resulting embryos, so these phenotypes can be used to detect mutations in the genes required for the polarized transport of mRNA within the oocyte. Now, for the first time, such a screen has identified a component of the cytoskeleton as an unexpected participant: on page 524 of this issue, Erdélyi et al.³ describe how mutants in non-muscle tropomyosin block almost all transport of *oskar* mRNA from the anterior of the oocyte to the posterior pole.

Tropomyosins form highly α -helical coiled-coil dimers that bind along actin filaments. In skeletal muscle, a troponin-dependent repositioning of tropomyosin inhibits the interaction between myosin and actin, thereby regulating muscle contraction. The role of tropomyosins in other cell types is less clear because these cells lack troponin. Non-muscle-tropomyosins are usually associated with the most stable actin structures within a cell, such as stress fibres, and *in vitro* studies suggest that these isoforms may both stabilize actin filaments and regulate the movement of myosin motors along them⁴.

This dual role is supported by genetic data from yeast. Mutations in one of the two tropomyosin genes in *Saccharomyces cerevisiae* cause a loss of stable actin cables and a defect in polarized growth

and secretion⁵, and the *Schizosaccharomyces pombe cdc8* tropomyosin gene is required for cytokinesis⁶. It is therefore a surprise to find that the first non-muscle tropomyosin mutants in a higher eukaryote should specifically impair the transport of a single mRNA, particularly as *Drosophila* seems to have only one non-muscle tropomyosin gene⁷. The original mutants isolated by Erdélyi et al.³ merely reduce the expression of non-muscle tropomyosin, but the lethal alleles generated by the imprecise excision of a P-element are likely to be null mutations. Nevertheless, these mutants do not block mitosis in the female germ line, nor the formation of stable actin structures such as the stress fibres in the nurse cells.

It seems, then, that in *Drosophila* at least, non-muscle tropomyosin has a much more limited and specific function than we expected. *Drosophila* may have another non-muscle tropomyosin gene, however, and this possibility needs to be ruled out before it can safely be concluded that non-muscle tropomyosins do not play a major role in actin organization.

The requirement for tropomyosin in *oskar* mRNA localization is also a surprise to those of us who work on axis determination in *Drosophila*, for several results have suggested that *oskar* mRNA is transported along microtubules to the posterior of the oocyte. First, *oskar* mRNA localization requires intact microtubules, as it is abolished by treating ovaries with the microtubule-destabilizing drug colchicine⁸. Second, dynein and a transgenic kinesin- β -galactosidase fusion protein, which are microtubule motors, both colocalize with *oskar* mRNA to the posterior of the oocyte, suggesting that the microtubules are polarized along this axis^{8,9}. Furthermore, when the oocyte develops a mirror-symmetric anterior-posterior axis, as in

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Subramanyan Chandrasekhar (1910–95)

SUBRAMANYAN Chandrasekhar, known to the world as Chandra, died on 21 August 1995. He is best known for his discovery of the upper limit to the mass of a white dwarf star, for which he received the Nobel Prize in Physics in 1983.

Chandra came from a highly educated South Indian family. His father, C. S. Ayyar, was a civil servant, attaining a high position with the Indian railways. The Ayyars had three sons and five daughters of whom Chandra was the oldest son, born on 19 October 1910 in Lahore, then part of British India. In 1916 the family moved to Madras where Chandra grew up.

Chandra was a brilliant student. At 15, he entered Presidency College, the most prestigious in Madras; in 1927 he started their physics honours course, graduating in 1930 at the top of his class. He read far beyond the curriculum, for instance about Fermi statistics, where he was most intrigued by Ralph H. Fowler's work on the constitution of white dwarf stars. This subject inspired him to write his first scientific paper, "Compton Scattering and the New Statistics", which was published in the *Proceedings of the Royal Society* in 1928. Upon graduation, on the basis of this paper, he was accepted as a research student by Fowler at the University of Cambridge.

He left Bombay on a boat on 31 July 1930. On the voyage, after overcoming his seasickness, he remembered Fowler's paper and decided to combine it with his knowledge of special relativity theory. To his great surprise, he found that this combination predicted that white dwarfs could only exist up to a certain limiting mass which depended chiefly on fundamental constants such as h , G and the mass of the hydrogen atom; the mass was about 1.45 times the mass of the Sun. England's two leading astrophysicists, Eddington and Milne, could not believe this result, and neither of them would recommend Chandra's paper for publication by the Royal Society. So Chandra sent it to the *Astrophysical Journal* in America, which published it in March 1931.

Chandra worked hard as a research student, and after he had taken his Ph.D., he was (to his great surprise) elected a fellow of Trinity College. Now feeling relaxed and more confident, he returned to the problem of white dwarfs. By a more complete calculation, he confirmed his earlier result: there is an upper limit to the mass of a white dwarf. He was invited to give a talk on this subject at the Royal Astronomical Society in January 1935. But after his lecture, Eddington stood up and rejected Chandra's results, not by scientific argument but by ridiculing the combination of special relativity theory with quantum statistics. Chandra was devastated.

Of course, Eddington was wrong. But his resistance to Chandra's mass limit was understandable: his life's work had been to show that every star, whatever its mass, had a stable configuration. It was generally (and correctly) believed that white dwarfs were

the end stage of stellar evolution, after their energy source was exhausted. Why should there be a limit to the mass of a star in its old age? Chandra appealed to physicists he knew—Rosenfeld, Bohr, Pauli. Unanimously, they decided that there was no flaw in his argument. But it took decades before the Chandrasekhar limit was accepted by the astrophysics community.

The paradox that 'normal' stars can exist with any mass whereas white dwarfs can only exist up to 1.45 solar masses is now understood as follows. Stars, in their evolution, go through a giant stage in which their radius may be hundreds of times larger than

IMAGE
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REASONS

originally. In this stage, the atoms at the surface are not strongly held by gravity, while there is strong radiation pressure from the inside. Some atoms, especially hydrogen, are blown off and the star gradually loses mass. Theory shows that stars up to 8 solar masses lose mass in this manner, ending up below the Chandrasekhar limit. None of this was known in 1935.

The limit also affects stars heavier than 8 solar masses. Matter in their central core evolves to iron by successive nuclear reactions. At this point, no further nuclear energy can be obtained, just as in white dwarfs. When the iron core grows to the Chandrasekhar mass, it collapses by gravitation into a neutron star, and the rest of the star is expelled, giving a type II supernova. Some white dwarfs accrete matter from the outside, and when their mass has grown to the Chandrasekhar limit, they also become supernovae, in this case type Ia. Chandra's theory is basic to much modern astrophysics.

Chandra had to look for a more permanent position, and accepted an offer from Yerkes observatory, part of the University of Chicago with which he remained associated for the rest of his life — first at Yerkes, later in Fermi's Research Institute on the main university campus. Before starting work there in January 1937, he made an

extensive visit to India and married Lalitha, his old friend from college days.

Chandra was an enthusiastic teacher who attracted students from all over the world. By the time of his retirement, he had guided over 50 students to their Ph.D.s. His own research covered nearly all branches of theoretical astrophysics; in his Nobel lecture, he said that his life's work fell into seven periods. At the culmination of each period, Chandra published a book collecting the insights he had gained; each covers its subject very thoroughly. *The Mathematical Theory of Black Holes* (1983) is truly formidable.

Chandra was a great supporter of Gandhi. But during the Second World War, he felt victory over Nazi Germany was the most important goal. Seeing it as his duty to contribute to the American war effort he began in 1943 to work half-time at the Aberdeen Proving Ground on shock waves. Later, he was invited to join the Los Alamos laboratory but, because of lengthy clearance problems, this did not come to pass. His feeling of duty was despite many indignities that he and Lalitha suffered because of their dark skin. Nevertheless, they felt American, and in 1953 were naturalized as American citizens, feeling their life would continue to be in America. This made Chandra's father very unhappy. He had tried repeatedly to get a suitable position for Chandra in India. But even if he had found one, what could have replaced the intellectual stimulation in America?

A year earlier, in 1952, Chandra had felt another call of duty. He believed there should be a national journal for astrophysics. Up to that time, nearly every major department of astronomy had its own publication. By long negotiations, he persuaded the American Astronomical Society and the University of Chicago jointly to publish the *Astrophysical Journal*, and Chandra, at great sacrifice of his scientific output, became its editor. In his 19 years as editor, Chandra transformed the *Astrophysical Journal* from a local publication into a world leader. He was, in his own words, dictatorial, and many astrophysicists complained because their papers were rejected. To continue at least some research, he rigorously enforced office hours: if a scientist phoned after the appointed closing time, he would answer, "The office of the *Astrophysical Journal* is closed."

I was always impressed by the depth and sophistication of Chandra's mind, and its capacity for retention which showed in his writings and speeches on any topic. His style was informed by the British authors of the late nineteenth and early twentieth centuries, and by Shakespeare. It was a pleasure to listen to one of his talks. In addition to style, he had the most perfect upper-class English accent I have heard.

Chandra was a first-rate astrophysicist and a beautiful and warm human being. I am happy to have known him.

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The North Pacific through the millennia

Lloyd D. Keigwin

CLIMATE in the North Atlantic region is well known to vary on timescales of millennia, with abrupt changes in sea surface temperature, high-latitude air temperature, pulses of ice rafting and changes in ocean ventilation all well documented. For the past several years the search has been on for similar millennial-scale variability in the Pacific region (and elsewhere) to determine the geographic extent of the sudden changes. Knowing the full geographical range of this variability in the linked system of ocean, atmosphere and cryosphere would be an important first step in finding its cause. Three recent papers describing North Pacific Ocean sediment cores¹⁻³, including one on page 510 of this issue², leave little doubt that the millennial-scale climate variability of the past 100 thousand years has affected most of the Northern Hemisphere.

Two of the papers present results from the California borderland basins. Using piston cores from the Tanner and San Nicholas basins, Thunell and Mortyn¹ report that not only did sea surface temperatures increase abruptly about 15 kyr ago at the end of the latest glacial episode,

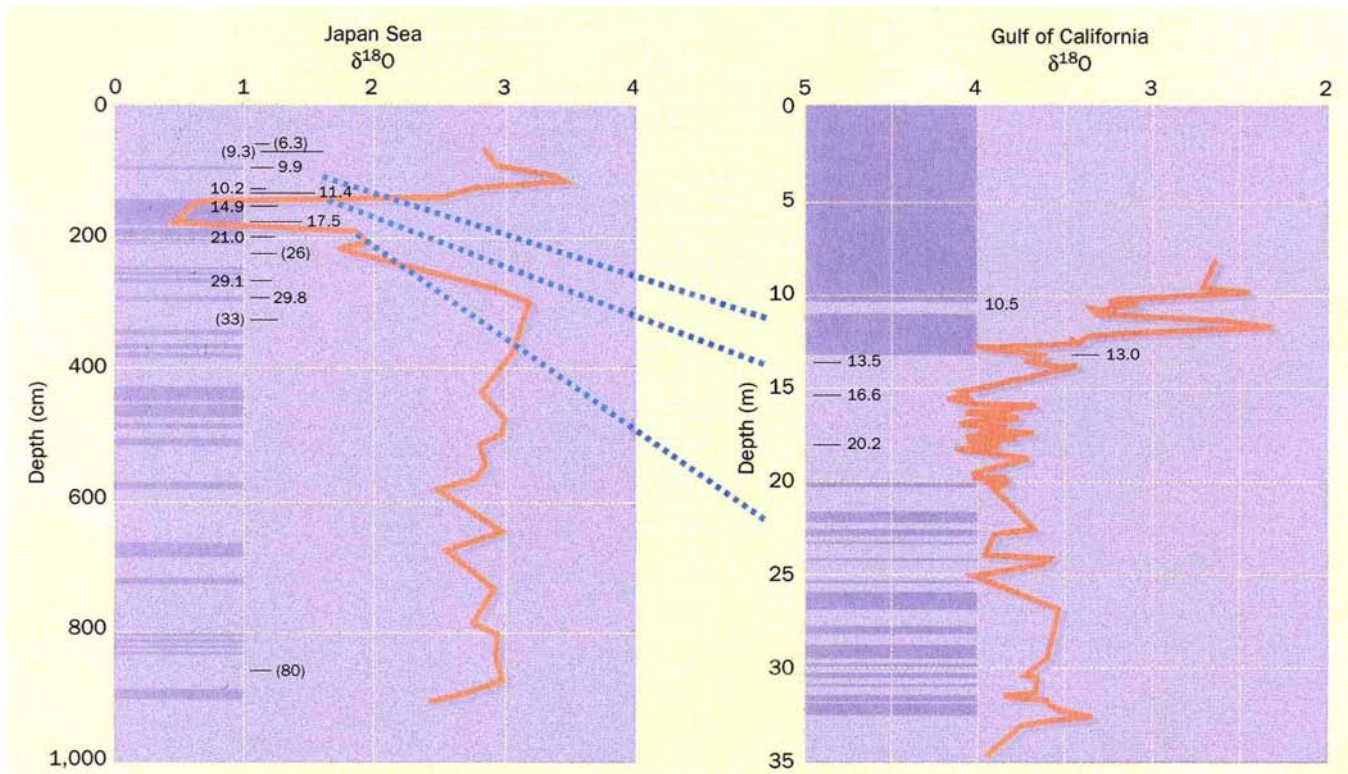
but they varied during the glacial period by as much as 6 °C. The proxy that they used for temperature, the polar planktonic foraminifera *Neogloboquadrina pachyderma* (left-coiling), changed both in absolute abundance and in abundance relative to warmer planktonic foraminifera. On this basis, it seems certain that there was considerable variability in sea surface temperature due to changes in the southward flux of cold subpolar water off southern California as far back as 40 kyr ago.

In their report on page 510 of this issue, Kennett and Ingram² use *N. pachyderma* as well as other proxies to describe the deglacial history of waters off southern California. Their core, from Santa Barbara basin, has rates of sediment deposition well in excess of a metre per thousand years, permitting them to focus on details of climate change not normally seen in the Pacific Ocean. Because of the high deposition rates, they discuss only the upper 32 m of their core, encompassing the past 20 kyr. Sediments in the Santa Barbara basin are frequently laminated, meaning that the oxygen content of the bottom water is sufficiently low (as it is today in the basin) that large burrowing organisms cannot

forage and mix out the seasonal changes in sediment type which give rise to the laminae. In contrast, glacial sediments in the basin are bioturbated, as are those of the brief cool event of about 12 kyr ago known as the Younger Dryas, which is *prima facie* evidence that at those times the oxygen content of bottom waters (at a depth of about 600 m) was higher.

As Kennett and Ingram point out, this result is nearly identical to observations in the Gulf of California at about the same depth, so there must have been a regional change in the ventilation of the eastern Pacific. The extent of climate change is about the same for the Younger Dryas event as it was for the last glacial maximum, except that the glacial depression of sea surface temperature was greater, and the climate changes in the Santa Barbara basin are virtually synchronous with climate events in the Greenland ice cores.

In the third paper, Kotilainen and Shackleton³ present evidence for millennial-scale variability in ice rafting of terrigenous sediment in a long sediment core from the far northwestern Pacific Ocean. Using measurements of core density, magnetic susceptibility and sediment geochemistry, they report that during the last glacial period there were many brief cold events marked by increased abundance of ice rafted debris (IRD). The origin of the debris is an important unknown. Does it reflect variability in mountain glaciers that reached sea level or was there an ice sheet



Lithostratigraphy (the darker shading shows laminated sediment) and foraminiferal $\delta^{18}O$ of a core from the Japan Sea¹⁰ and the Gulf of California¹³. Dating is by the accelerator radiocarbon method for the Japan Sea¹¹ and the Gulf of California¹⁴; additional dates in

parentheses are ages of volcanic ash layers¹⁰. Note that the depth scales and the $\delta^{18}O$ scales differ between the two cores. ($\delta^{18}O = (^{18}O/^{16}O)_{\text{sample}} / (^{18}O/^{16}O)_{\text{standard}} - 1$, where standard is Pee Dee belemnite. Values are per mil.)

nearby? Although the available chronology for their core is not good enough to correlate North Pacific IRD events explicitly with North Atlantic IRD events⁴ or Greenland air temperature⁵, their conclusion is that the frequency of climate variability is probably the same in the North Atlantic and the North Pacific.

So what does this trio of papers tell us in aggregate about climate change in the North Pacific region? First, we now know that deglacial climate oscillations in the eastern North Pacific are regional, and synchronous with those of the North Atlantic. Second, we know that climate variability before the glacial maximum extends clear across the North Pacific, and it is likely that these events correlate with the short-term 'Dansgaard-Oeschger' changes in Greenland air temperature⁵ and similar events in the surface of the subpolar North Atlantic⁴. Third, we know that somehow these surface ocean changes are linked to ventilation at intermediate depths in the North Pacific during the past 20 kyr.

Other work over the past few years may help clarify this picture. For example, oscillations in the lamination of the Santa Barbara basin sediments continue back beyond the glacial interval and appear to be directly correlated with the Greenland temperature proxy data⁶. This suggests that the relationship between North Pacific ventilation and surface climate must be strong for at least a dozen cold episodes similar in scale to the Younger Dryas. And geochemical data on benthic foraminifera from the northwestern Pacific indicate lower nutrient levels, and hence better ventilation during glacial maximum conditions^{7,8}.

The fact that the far northwestern Pacific is the source for intermediate depth waters today⁹ points to that region as a likely source of variability during glacial times. For now, unfortunately, we lack any evidence of ventilation changes in the source region on millennial timescales. However, the nearby Japan Sea is known to be very sensitive to changes in its surface hydrography, and sediment cores are alternately laminated and non-laminated¹⁰ (this sea is isolated enough from the open ocean that the oscillations are not directly related to the IRD events recorded in ref. 3). This sets up an interesting lithological oscillation on opposite shores of the North Pacific (see figure). When climate is harsh the dissolved oxygen content of the deep Japan Sea decreases below some critical level, perhaps because the ice cover extends over regions of convection or because of small changes in the fresh water balance. Under those conditions, Japan Sea sediments become laminated.

In the extreme case of maximum glaciation, a fall in sea level partially isolates the basin even more than today from the open

sea, and the $^{18}\text{O}/^{16}\text{O}$ ratio and salinity of surface water decrease (see figure). The severe climate which promotes oxygen depletion in the Japan Sea and better ventilation or more intermediate water production in the open northwestern Pacific may result in nonlaminated sediment far downstream in the Gulf of California and the Santa Barbara basin^{2,6}. Thus, just as thermohaline circulation and surface ocean conditions are tightly coupled in the North Atlantic on millennial timescales, the same may be true in the North Pacific.

The exact mechanism for linking North Atlantic and North Pacific climates on millennial or 'sub-orbital' timescales is now the central question. Of the numerous possible causes, most authors consider some sort of atmospheric teleconnection^{1-3,12}. Thunell and Mortyn suggest that large changes in the height of the Laurentide ice sheet might have steered the jet stream and the southward flow of subpolar waters off southern California, but there is little evidence for such large changes in ice volume as frequently as every few thousand years. Kennett and Ingram propose a global change transmitted through the atmosphere, but they do not speculate on its origin. Likewise, Kotilainen and Shackleton consider the leading contender to be major fluctuations in air temperature. Atmospheric forcing has been directly implicated by Porter and Zhiseng¹², who linked the winter monsoon, dust accumulation in China and surface ocean cooling in the North Atlantic. Whatever the forcing for synchronous changes in Northern Hemisphere climate on geologically short timescales, which will surely be the focus of much research, palaeoceanographers deserve credit for having made great strides during the past few years in extracting high-resolution climate signals from North Pacific sediments. □

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Clean burning

Air pollution is everywhere these days. Traffic fumes pour into city streets while waste gases eddy round industrial estates. 'Sick' buildings and offices fume with vapours released from their furnishings and equipment, while clubs and pubs accumulate headache human 'fug'. Nearly all air contaminants burn, or are decomposed by combustion. But how to burn such a dilute fuel?

One way is to pre-heat it. Counter-current combustion warms the incoming air by close contact with outgoing combusted air, and can burn very dilute vapours. Another way is to use a catalyst, to lower the burning temperature (exhaust catalysts work this way). Daedalus is now combining these two approaches, with the aid of aerogel technology.

Aerogels are light, microporous, inert solids, so full of molecular holes that they are not much denser than a gas. They can be amazingly good heat insulators. Daedalus's novel catalytic combustor is an aerogel sandwich. A thin film of aerogel is impregnated on one side with a suitable combustion catalyst, and two such films are butted together with their catalyst layers on the inside.

Put this structure in polluted air, and the vapours will diffuse inwards through the aerogel to be burnt on the central catalyst. The resulting hot purified air will diffuse out again the same way, transferring its heat in counter-current fashion to fresh incoming air. Thanks to the microporous but wonderfully heat-insulating aerogel, the central catalyst will stabilize at perhaps 250 °C while the surface of the sandwich is almost at room temperature. The aerogel combustor will be 'lit' by a brief pulse from an electric heater in one corner. Combustion will spread slowly from that point until the whole sheet is working. Thereafter, it will purify the air around it continuously, with no further attention.

The aerogel combustor will bring a breath of fresh air to every walk of life. On the walls and ceilings of offices and workshops, public halls and washrooms and private dwellings, it will burn up stale human effluvia and machine fumes alike, giving a clean, crisp, fresh atmosphere. On hoardings, street signs and fences, and as cladding for buildings and road-tunnel roofs, it will burn up exhaust gases as fast as they are generated. Sadly, an aerogel road surface would be rapidly wrecked by the passing wheels, but a lot of town air could be cleaned overhead by means of cheerful, waving, aerogelled flags and bunting. On a smaller scale, Daedalus is dreaming of aerogel wallpaper for smelly kitchens, and aerogel underwear to burn up the dreaded BO at source.

David Jones

Cretaceous mushrooms in amber

SIR—The remains of two gilled mushrooms were recently discovered in Turonian (90–94 million years old, mid-Cretaceous) amber from central New Jersey, USA¹. (The amber was found by G. R. Case, P. D. Borodin and J. J. Leggett in November 1994.) This is more than three times older than the previously reported oldest gilled mushroom, *Coprinites dominicana*², from Dominican amber, which was recently dated at 25–30 million years³ (revising an original estimate of 40 million years).

Gilled mushrooms are members of the holobasidiomycetes, a morphologically diverse group of fungi whose fruiting bodies also include puffballs, polypores and stinkhorns. Most holobasidiomycete fruiting bodies are ephemeral, and so their fossils are exceedingly rare. Certain holobasidiomycetes, such as wood-rotting polypores, produce tough, perennial fruiting bodies which might be expected to produce fossils more readily than fleshy mushrooms. Indeed, the oldest clearly holobasidiomycetous fossil is that of an apparently perennial polypore, *Phellinites digustoi*, from the Jurassic⁴. Basidiomycete hyphae with diagnostic clamp connections are known from the Pennsylvanian⁵, but without knowledge of fruiting-body morphology, or key anatomical and ultrastructural characters, it is impossible to tell whether these were produced by a holobasidiomycete or by some other group of basidiomycetes, such as the jelly fungi (heterobasidiomycetes).

The New Jersey fossils are unquestionably holobasidiomycetes. One of the specimens (AMNH NJ-90Y; Fig. 1a) is a nearly complete mushroom which has an intact cap with distinct gills and a central stalk. The other specimen (AMNH NJ-90Z) consists of a wedge-shaped fragment of a cap. Both are minute; the cap of AMNH NJ-90Y is 3.2 mm in diameter. Because they occurred in the same piece of amber, it is most likely that both specimens were produced by the same mycelium. Spore fragments and casts indicate that the fossil mushrooms had smooth, elliptical spores (Fig. 1b,c). Morphologically, the fossil mushrooms bear a strong resemblance to the extant genera *Marasmius* and *Marasmiellus*⁶, which are common, widespread decayers of leaf litter and wood. Bark fibres and leaves embedded in various amber pieces from the site where the fossil mushrooms were found suggest that the mushrooms had been growing on a member of the Cupressaceae (the 'cedar' family). A formal taxonomic diagnosis of the fossil mushrooms will be presented elsewhere.

The discovery of these fossils does not change the estimate of the minimum earliest date for the divergence of the holobasidiomycetes, which is still set by *Phellinites* from the Jurassic⁴. Nevertheless, it demonstrates that by the mid-Cretaceous, holobasidiomycete morphological diversity already encompassed forms as dissimilar as robust, perennial, conk-like polypores (*Phellinites*) and

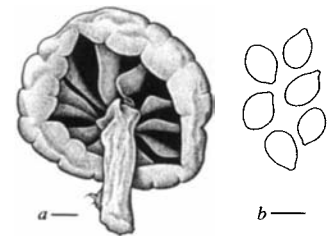


FIG. 2 Sketch of fossil mushroom micro- and macromorphology. a, Habit, based on Fig. 1a. b, Spore morphology, based on Fig. 1b, c. Scale bars: a, 0.5 mm; b, 6 μ m.

minute, gilled 'toadstools'. This is consistent with the conclusions of Berbee and Taylor⁷, who estimated the radiation of the holobasidiomycetes to have begun around 200 million years ago. The New Jersey fossils provide another calibration point for basidiomycete molecular clocks.

The real significance of the New Jersey fossils may lie in their impact on our conception of the pace and mode of morphological evolution in mushroom-forming fungi. If the New Jersey fossils are, in fact, closely related to *Marasmius* and *Marasmiellus*, this suggests a strong conservation of form in this lineage for many millions of years. In contrast, recent molecular systematics studies have shown that evolution in diverse holobasidiomycete clades has been marked by abrupt morphological transformations, including those between mushrooms and false truffles⁸, agaricoid and secotioid fungi^{9,10}, or polypores and gilled mushrooms¹¹. Despite many profound evolutionary changes in holobasidiomycete fruiting-body form, including repeated evolution of gilled mushrooms¹¹, the discovery of these new fossils suggests that certain extant morphologies may be of very ancient origin.

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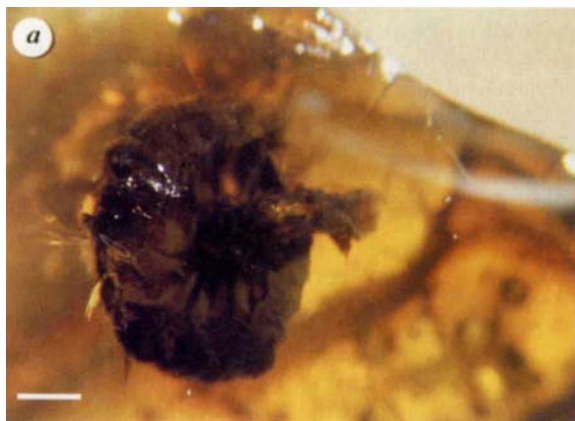
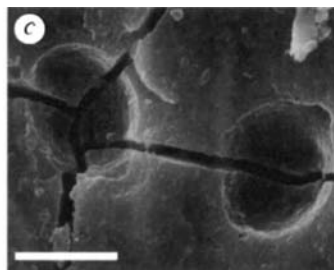
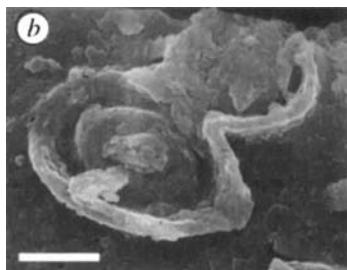


FIG. 1 Fossil mushroom from New Jersey amber. a, AMNH NJ-90Y. View from underneath cap, showing radial gills and central stalk. b, c, Scanning electron micrographs of spore fragments (b) and spore casts (c) from AMNH NJ-90Z. Scale bars: a, 0.5 mm; b, 2 μ m; c, 5 μ m.



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The real threat to saiga antelopes

SIR—The saiga antelope has been the subject of recent heavy press coverage, catalysed by the publication of a report into its status¹. The tenor of the coverage has been that the species is on the brink of extinction due to poaching for its horns (for example, ref. 2). It is claimed that the species has declined alarmingly since the break-up of the Soviet Union, when illegal horn exports increased dramatically. Here we present up-to-date data on the status of the species which do not support the claim that the population has declined. The data do give serious cause for concern, however, about the low proportion of adult males in all four saiga populations, leading to the possibility

of a sudden crash in numbers.

Four populations of the subspecies *Saiga tatarica tatarica* exist, three in Kazakhstan and one in Kalmykia (Russian Federation). The subspecies *S. t. mongolica* is found in Mongolia in very small numbers. The populations of *S. t. tatarica* declined to very low levels by 1920, but recovered under protection and have been hunted and managed primarily for meat since 1950. Given this history, a healthy trend would be for the population to grow rapidly, reach high levels and then decline to a relatively stable level under sustainable exploitation. Thus, the fact that a population is now lower than at its peak is not necessarily a cause for concern if the population is stable at more than 50% of

carrying capacity. The total population has varied around 1 million since regular annual censuses were established in 1978 (Fig. 1a). The Ustyurt and Ural'sk populations have increased slightly since 1975, the Betpakdalinsk population fell in 1974–75 and since then has shown no clear trend. In Kalmykia, the population rose to a peak in 1971–78, then declined

until 1987. The decline stabilized during 1988–93 (with the exception of 1991). These population figures give no indication of a plunge in numbers after 1990.

A major concern about selective poaching for horns is that the proportion of adult males in the population may become very small. The data indeed show that the proportion of adult males in the population declined over the period 1988–93 in Kazakhstan and fell sharply in 1992 in Kalmykia (Fig. 1b). A lack of males can affect the population dynamics of a species in various ways³. The saiga is a polygynous rutting species and thus the most likely mechanisms are juvenile mortality caused by lengthening of the parturition period and reduced female fertility caused by a lack of mating opportunities. Female fecundity is strongly affected by the unpredictable climate in the region and so it is hard to distinguish trends. However, no clear recent trend is visible in the data (Fig. 2). Models have shown^{3,4} that the effect of a lack of males on population dynamics can be strongly

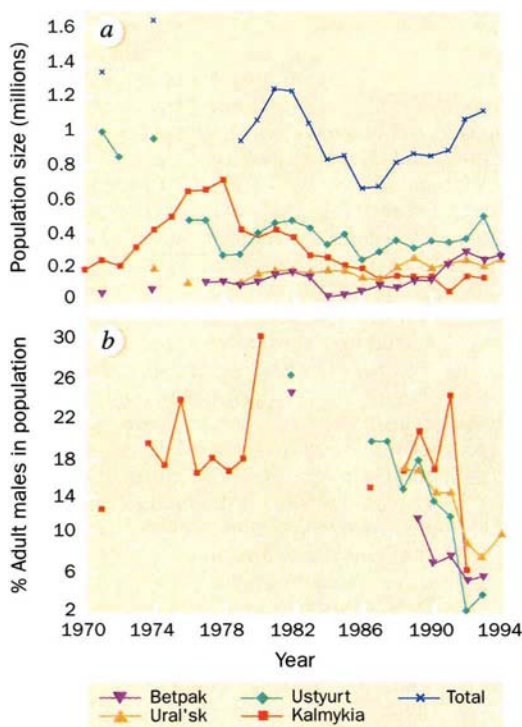


FIG. 1 a, Estimates of the size of *Saiga tatarica tatarica* populations, 1970–94. Data for Kalmykian subpopulation are from Teer *et al.*⁶; data for Kazakhstan include the most recently available estimates. Betpakdalinsk is in central Kazakhstan, Ustyurt between the Caspian and Aral seas, and Ural'sk to the north of the Caspian Sea in west Kazakhstan. Kalmykia is to the west of the Caspian Sea. The standard errors for the Kalmykian estimates are between 11 and 14% in all years except 1991 (22%) and 1993 (34%), but standard errors are not available for the Kazakhstan estimates, so caution is required in their interpretation. b, Proportion of adult males in the four sub-populations, 1969–94. Data for Kalmykia and for Kazakhstan up to 1987 are from observations of herds, Kazakhstan data after 1988 are from hunted specimens. Kalmykian data are from Teer *et al.*⁶. The change in collection methods means that these data are not comparable after 1988. In particular, there has been no test of the reliability of the ostensibly unselective hunting procedure. Given this proviso, the data do still show a worrying trend in sex ratio in all populations.

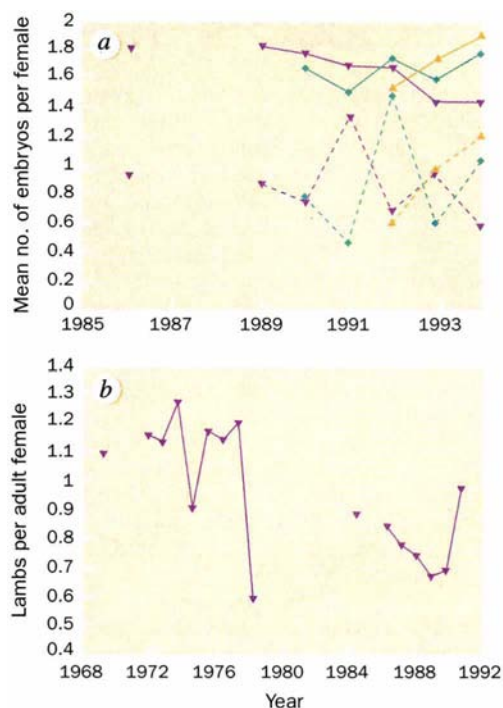


FIG. 2 a, Mean number of embryos per female in the three Kazakhstan populations, 1986–94. Juveniles are shown as a dashed line, adults as a solid line. b, Mean number of lambs per female in Kalmykia, 1969–93. Data are from Teer *et al.*⁶. Note that the Kazakhstan data are expressed in terms of embryos per female and thus only show fertility rate, whereas the Kalmykian data, presented as lambs per female, comprise both fertility and perinatal mortality of juveniles. Key as for FIG. 1.

nonlinear, and thus that the lack of a clear correlation between female fecundity and sex ratio should not be taken as evidence that the populations are secure.

Although interest in the saiga antelope by the conservation community is welcome, it is inadvisable to 'cry wolf' if there is no immediate threat to the saiga from male-biased poaching. As these data have shown, the lack of adult males has yet to make a strong impact on population size. The concern must be, however, that, because the effects of a lack of adult males are likely to be nonlinear, any population decline due to this factor will be very rapid. Having said this, it is important to guard against using poaching as a convenient and relatively easily tackled scapegoat that can mask other important threats facing the saiga. In particular, the steady decline of the Kalmykian population since the late 1970s could be attributed to accelerating desertification in the region, caused by more intensive human use of the area⁵,

Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. Priority will be given to letters of fewer than 500 words and five references.

and thus to the reduction in availability of suitable habitat. If this is a major threat to the saiga, it too should also be tackled urgently. The Mongolian subspecies is apparently not affected by poaching, but is urgently in need of protection because of its very small size (about 336; ref. 1). Concentrating solely on the problem of poaching sidelines this population.

If poaching is not yet affecting population sizes, one reason may be the recent reduction in legal hunting, and particularly in the proportion of males in the legal hunt. Thus, a key issue not being addressed is the acquisition of large revenues from saiga poaching by commercial traders, revenues which are being denied to the government cooperatives and local people who bear the costs of saiga preservation. As horns did not form a large proportion of the cooperatives' revenues before 1990, this lost opportunity goes unacknowledged. If saigas are to continue to form a valuable and respected resource for the residents of their range areas, the revenues should be accruing to the correct parties.

Clearly, poaching must be brought under control rapidly both to secure the future of the population and to allow legal harvests to continue. However, we would like to present the story of the saiga antelope as a positive one. It is rare to find a species that reached near-extinction but recovered in 30 years, and which was then harvested for 40 years in an apparently sustainable way. The products of that harvest were both sold to local people as cheap meat and went to support the general economy. The population was well researched and regularly censused. Only in the last five years has the financial situation for the management authority become problematic and poaching a serious problem. This rare example of a successful, sustainable management system should be supported by the international community before it is lost, and managers should be enabled to use the new markets for horn effectively, to secure the future of the saiga antelope and its users.

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Touching the phantom limb

SIR — Despite a vast clinical literature on phantom limbs (for example, refs 1, 2), there have been no experimental studies on the effect of vision on phantom sensations. We used a mirror to resurrect the phantom visually in order to explore intersensory effects³.

Nine arm amputees were studied (see table). A tall mirror was placed vertically on the table, perpendicular to the patient's chest, so that he could see the mirror reflection of his normal hand 'superimposed' on the phantom. In the first seven patients, when the normal hand was moved so that the phantom was visually perceived to move in the mirror, it was also 'felt' to move; that is, a vivid kinaesthetic sensation emerged. (These sensations could not be evoked with the eyes closed.) In patient D.S., kinaesthetic sensations were evoked even though he had not experienced movements in the phantom for the preceding 10 years. Repeated use of the mirror over a 3-week period (15 min per day) resulted in a permanent 'telescoping' of the hand in this patient.

In six of these patients, a similar revival of movements in the phantom occurred if the experimenter's hand was substituted for the patient's normal hand. No such effects were seen in four normal 'control' subjects given identical instructions.

Five patients (R.L., P.N., R.T., B.D. and J.P.) experienced painful involuntary 'clenching spasms' of the phantom hand ("As though the nails were digging in the phantom," as J.P. told us). Remarkably, in four of them, the spasms, which normally lasted for an hour or more, could be relieved immediately on looking into the mirror and opening 'both' hands simultaneously. (R.T. compared 8 eyes-closed/eyes-open trials; P.N., 8 trials; R.L. 6 trials and J.P. 12). No eyes-closed trials were effective in relieving spasms.

When motor commands are sent from the premotor and motor cortex to clench the hand, they are normally damped by error feedback from proprioception. In a phantom, such damping is not possible, so the motor output is amplified further and this outflow itself may be experienced as a painful spasm. Visual feedback from the mirror may act by interrupting this loop.

The elimination of spasms was unequivocal in all four patients. Interestingly, the associated pain also disappeared. Given the notorious susceptibility of pain to 'placebo', however, double-blind experiments would be needed to determine whether the effect on pain is a specific consequence of the visual feedback.

In two patients (J.P. and L.C.), touching the normal hand evoked precisely localized

CLINICAL DETAILS OF PATIENTS TESTED

Patient	Age	Pathology	Location	Time of testing
J.P.	31	Self inflicted amputation	To right forearm 5 cm below elbow	5 months after amputation
R.L.	56	Melanoma infiltrating brachial plexus	Right upper limb disarticulation at shoulder 1 year after onset of melanoma	2 months after amputation
P.N.	48	Arm crushed in car accident	Left arm 8 cm below elbow	7 months after amputation
R.T.	55	Sarcoma infiltrating ulnar nerve	Left arm 6 cm above elbow	7 months after amputation
P.N.N.	40	Airplane propeller cut off arm	Right arm above elbow	8 years 3 months after amputation
D.B.	23	Car accident, crush injury	Left arm, disarticulation of shoulder	3 years after amputation
D.S.	28	Brachial plexus avulsion	Left above-elbow amputation 1 year after avulsion	9 years after amputation
B.D.	29	Brachial plexus avulsion	Right above-elbow amputation 2 years after avulsion	3 months after amputation
L.C.	23	Crush injury following train accident	Right forearm below elbow	19 days after amputation

All patients underwent a thorough neurological evaluation by one of us (V.S.R.) to rule out central nervous system pathology and to ensure that their 'mental status' was normal. None of the patients (except L.C.) could produce voluntary movements in the phantom. When D.S., J.P. and D.B. were touched on the lower-face region, ipsilateral to the amputation, sensations were referred to the phantom fingers, as occurred in some of the patients we had previously studied^{4,5}. D.S. also had magnetoencephalographic evidence of 'remapping' in the cortex^{5,6}. B.D. did not show any inter-manual referral of sensations whether or not he used the mirror box. Also, he could not generate any movements in the phantom, whether or not he used the box, and there was no relief from pain. ("It's frustrating, doctor. I can see it move; I want it to move; but it doesn't feel like it's moving!") Thus, the procedure may not work on all patients and the reasons for the variability remain to be explored.

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touch sensations in exact mirror-symmetrical locations in the phantom. In two additional patients (R.L. and D.B.), such effects occurred only if the patient simultaneously 'saw' the phantom being touched — a curious form of 'synaesthesia' (14 out of 14 trials). Vibration was also referred intermanually in all four patients, but there was no referral of pain, heat or cold. For example, if patients J.P. or L.C. dipped their intact hand in a jar of cold water with ice cubes, they responded that they felt the 'cubes' in the phantom, but not the cold. These effects are not due to suggestion or confabulation for three reasons. First, touch and vibration were referred but not temperature or pain, and this was consistent across patients. Second, the patients experienced a latency of 2–4 s before the sensation was felt in the phantom. Third, the patients often expressed considerable surprise when they noticed these effects.

Even in normal individuals, the two hands may be linked in the brain as a result of frequent co-activation. Hence, sensory input from the left thumb might project not only to the right hemisphere but — by unidentified commissural pathways — to mirror-symmetrical points in the other hemisphere. This latent input may ordinarily be too weak to express itself, but when the right hand is amputated this input may become either disinhibited or progressively strengthened, so that touching the left hand evokes sensations in the right hand as well. The reason pain and temperature are not referred may be that there are no commissural pathways concerned with these modalities. In R.L. and D.B., however, the reactivation may not reach threshold amounts unless visual 'confirmation' is also provided (cells with bimodal receptive fields⁷ may be involved).

Our technique lends itself readily to brain-imaging studies. There must be considerable latent plasticity in the adult human brain. Precisely organized new pathways spanning the hemispheres can emerge in three weeks or less. Also, there must be great interaction between vision and touch, and so the strictly modular, hierarchical model of the brain currently in vogue must be replaced with a dynamic, interactive model in which 're-entrant' signalling⁸ plays an important role.

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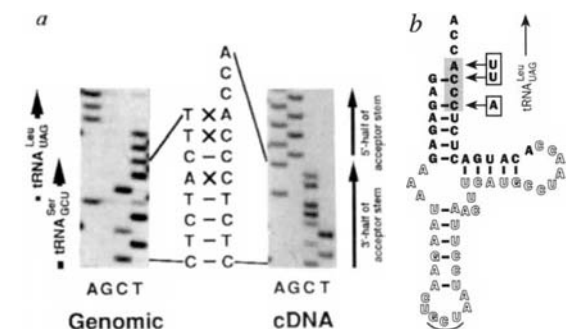
tRNA editing in metazoans

SIR — RNA editing, the modification of a transcript such that it differs from that inferred from its genomic sequence, is a common phenomenon in organelles of plants and unicellular organisms¹. In metazoan mitochondria, polyadenylation is used to create termination codons² and may be involved in the editing of several

acceptor stem had been replaced by cytidine residues (see figure). Curiously, one mismatch remained unchanged. In addition, the discriminator base had changed from the genomic uridine residue to an adenine residue. Because the complete sequence of the mitochondrial genome of the platypus is known⁶ and import of cyto-

plasmic tRNAs into mitochondria is not believed to occur in vertebrates⁷, the tRNA gene is unlikely to represent a pseudogene; more probably, an RNA-editing mechanism changes the primary sequence of the tRNA post-transcriptionally.

RNA editing has recently been found to affect aminoacylacceptor stems of mitochondrial tRNAs in a protozoan (*Acanthamoeba castellanii*)⁸ and a land snail (*Euhadra herklotsi*)³. In the latter, the 13 changes observed in three tRNA species are similar to the three changes found in the platypus in that they involve events in the 3' parts of the aminoacylacceptor stems. A further similarity between the platypus and land snail is that the genes of the tRNAs affected overlap with downstream genes encoded on the same strand. Such overlaps also exist in the mitochondrial



a, cDNA and gene sequences of the platypus mitochondrial seryl-tRNA anticodon GCU (tRNA^{Ser}_{GCU}). Only relevant parts of the autoradiographs of sequence ladders are shown. Differences between the genomic and cDNA sequences are indicated by crosses, identities by dashes. b, Inferred secondary structure of platypus mitochondrial tRNA^{Ser}_{GCU}. The region used for primers in cDNA synthesis and polymerase chain reaction (PCR) are indicated by outlined characters. The region that in the gene overlaps with the downstream tRNA for leucine is indicated by the shaded background and the genomic versions of the edited nucleotide positions are boxed. tRNA circularization, cDNA synthesis, PCR and sequencing were carried out as in ref. 3. RNA of platypus and the clone carrying the platypus tRNA^{Ser}_{GCU} gene were gifts from A. Janke. The primer used for cDNA synthesis was 5'-GGATTAGCAGTCTTATT-3' (platypus-S-FW). For amplification, this primer and 5'-TTAACTTCATGCCTAAC-3' (platypus-S-RV) were used.

transfer RNAs in land snails³. In addition, another RNA-editing mechanism changes an anticodon of a tRNA in marsupial mammals^{4,5}. Here we show that the tRNA of a monotreme mammal is edited at three positions and that RNA editing is likely to be common in metazoan mitochondria.

The genomic sequence for the mitochondrial seryl-tRNA anticodon GCU of the platypus (*Ornithorhynchus anatinus*)⁶, a monotreme mammal, has two mismatches and one G·U base pair in its predicted aminoacylacceptor stem. In contrast, other features of the inferred tRNA structure are common to other seryl-tRNAs (data not shown). To investigate whether these unusual features are corrected by an RNA-editing mechanism affecting the 5' or 3' halves of the acceptor stem, RNA was circularized³ by T4 RNA ligase before synthesis of complementary DNA. The acceptor stem was then amplified, cloned and the nucleotide sequence of more than 20 clones determined.

It was found that one mismatched adenosine residue and the uridine residue of the G·U base pair in the 3' part of the

genomes of various metazoans, for example, human, mouse and chiton^{9–11}. Thus, the finding of tRNA editing in a land snail as well as the platypus makes it likely that an RNA-editing activity is involved in the processing of the transcripts in many, if not all, metazoan mitochondria.

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Theories of nothing

David Papineau

The Mangle of Practice: Time, Agency and Science. By Andrew Pickering. University of Chicago Press: 1995. Pp. 281. £35.95, \$45 (hbk); £14.25, \$17.95 (pbk).

ANDREW Pickering is a professor of sociology, criticism and interpretive theory at the University of Illinois, and a leading figure among contemporary sociologists of science. His first book, *Constructing Quarks* (1984), charted the steps leading to the acceptance of quarks as the fundamental units of matter. This was widely acclaimed as a theoretically sophisticated history, not least by some of the physicists involved. Their only caveat was that they didn't understand the bits at the beginning and the end, where Pickering seemed to be saying that the quark story was all made up, or "constructed". I suspect that such readers will find even more to puzzle about in this new book, which contains far more philosophy and rather less history.

There are many differences among contemporary sociologists of science, but one aim uniting most of them is a concern to expose the realist pretensions of science. They reject the idea that experiment and debate allow science to home in on the true mechanisms behind the appearances. They maintain instead that the choice of theories is invariably the product of nonrational factors such as ideological correctness or the manoeuvring of power groups within the scientific community.

Pickering originally endorsed an extreme version of this view, holding that experimental results never have any influence on the thinking of scientists. In *The Mangle of Practice* he can be seen inching his way closer to common sense. He now recognizes that experiments will produce only some results and not others, and that this often forces scientists to rethink their theoretical assumptions. More generally, he argues that science-related projects of all kinds, from mathematical analysis to the introduction of new industrial technologies, are shaped by the need to solve problems that emerge in practice. Or, as he puts it, scientific culture is reconfigured in an emergent posthumanist way as it gets "mangled" by a dialectic of resistance and accommodation.

Once one works out what Pickering means, there is nothing particularly contentious about this general explanatory scheme, and in his central sections he applies it to four case studies: the evolution of the bubble chamber, a series of Millikan-style quark-seeking experiments, Hamilton's formulation of the theory of quaternions and the introduction of numerically controlled machine tools to a General Electric factory in Massachusetts. In each case he shows how some initial

scheme gets remodelled because it doesn't always work as planned.

If there is an objection to Pickering's explanatory scheme, it is that it seems able to fit any dynamic process, not just those within scientific culture. After all, any sequence of changes can be viewed as a series of deflections from some initial tendency. This thought occurs to Pickering too. In a rather wild-eyed postscript, he follows his argument where it leads, and recommends that we view "the evolution of the universe as a whole — of inorganic as well as organic matter — as evolving within fields of agency in dialectics of resistance and accommodation". The *Mangle of Practice* becomes a Theory of Everything, and yields a "physics without quarks" and a "biology without genes".

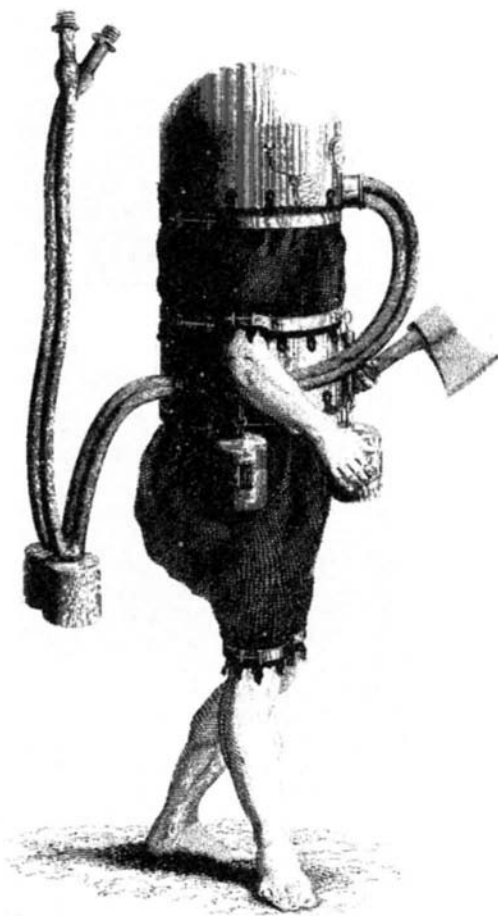
It is perhaps unfair to pick on an afterthought that Pickering himself characterizes as hubristic and speculative. A more serious question concerns his blanket

rejection of scientific realism. It is of course not unknown for over-zealous groups of scientists to dress up ephemeral speculations in the cloak of scientific objectivity. But Pickering, along with most of his sociological colleagues, is committed to the far less sane view that *no* scientific theory ever gets reality right — not even the atomic theory of matter, say, or the theory that measles is caused by a virus.

It is puzzling why Pickering holds this view. Nothing in this book amounts to an argument for it. But there is a revealing section in his first chapter, which suggests that his real motivation may be insecurity about the professional standing of sociology. He quotes an objection by two other sociologists to the idea that experimental results can be important in explaining scientific developments. The worry is that this would require sociologists to defer to scientists for an understanding of the workings of the relevant experiments, and this is not something "any sociologist with a shred of self-respect" would want to do, as Pickering puts it.

Pickering's response to this objection is that sociologists shouldn't defer to a scientific understanding of the experiments, because they will normally be studying *past* scientists who haven't yet agreed on any such understanding. Even on Picker-

TESTING the water: this "diving machine" was first described in the second volume of *The Philosophical Magazine* in October 1798. Both the article and the picture are reproduced in *Science in the Making — Volume 1: 1798–1850* edited by E. A. Davis, the first of three anthology volumes on the development of science as chronicled by papers in *The Philosophical Magazine*. Originally published to embrace a broad spectrum of topics in natural philosophy, the journal today is devoted exclusively to the physics of condensed matter. Taylor and Francis, £59.95.



ing's own terms this response scarcely makes sense, given that it doesn't explain why it would be wrong to defer to *present* scientists who have reached agreement on how the experiments work. But even more worrying is his taking the objection seriously in the first place. After all, why should it count against an independently plausible thesis that it threatens the self-respect of sociologists of science?

I suspect that this kind of professional anxiety accounts for many of the moves made by Pickering and his colleagues in the sociology of science. They resist views, like scientific realism, which they see as threats to their professional status. Unfortunately, this kind of thinking is unlikely to carry any weight outside the sociological profession. Outsiders may wish to sympathize with the sociologists' lack of self-confidence, but they will have little reason to be persuaded of their views. □

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The healing arts

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Genes, Blood and Courage: A Boy Called Immortal Sword. By David G. Nathan. Harvard University Press: 1995. Pp. 276. \$24.95, £15.95.

THE hippocratic oath, always taken more seriously in the United States than in the United Kingdom, enjoins physicians to refrain from discussing the ills of their patients in public. There has never been, however, a shortage of physician-writers who used their medical background to dramatize the relationship between doctors and their patients. The names of A. J. Cronin, Francis Brett Young and Arthur Conan Doyle quickly come to mind. Somerseset Maugham, in his last major work *Summing Up*, reflected on the importance of his clinical experience in his writing:

I saw how men died. I saw how they bore pain. I saw what hope looked like, fear and relief; I saw the dark lines that despair drew on a face; I saw courage and steadfastness. I saw faith shine in the eyes of those who trusted in what I could only think was an illusion, and I saw the gallantry that made a man greet the prognosis of death with an ironic joke because he was too proud to let those about him see the terror of his soul.

The self-imposed convention of silence was dramatically breached in 1962 when Lord Moran decided to discuss Winston Churchill's medical problems. His book, *Churchill: The Diaries of Lord Moran*,

evoked a barrage of criticism from the profession, as Richard Lovell, in his recent biography, *Churchill's Doctor*, makes abundantly clear.

During the past 30 years, however, the perception that doctors should remain silent about the diseases of their patients has altered. Perhaps the most startling medical revelations related to the changing state of President Eisenhower's bowels, which held the American public suitably enthralled. This was in marked contrast to Lord Dawson of Penn's laconic bulletin on King George V: "The King's life is moving peacefully towards its close".

In *Genes, Blood and Courage*, the distinguished paediatric physician and clinical investigator David Nathan provides a riveting account of a young boy who was first seen by Nathan at the Children's Hospital in Boston at the age of 10. Astute clinical and haematological investigation rapidly disclosed that Dayan Saif (a pseudonym to preserve strict confidentiality), of Arab extraction, was suffering from thalassaemia major, an inherited disease notoriously difficult to treat and one that often leads to an early death. When the book ends, Dayan is 30 and has weathered the physical and psychological vicissitudes of his disease by Nathan's rare combination of high professional competence and sustained compassion.

The story told so well is a moving account of the 30-year struggle to keep Dayan alive physically and spiritually in the face of a prognosis of grim proportions. It is one of the many virtues of Nathan's book that he uses Dayan as a poignant example of the increasing importance of the molecular revolution in the treatment of human diseases, particularly those such as thalassaemia that are of genetic origin.

Nathan tells the dramatic story of how the development of a new iron-chelating agent made possible the elimination of excess iron from Dayan's body, the inevitable consequence of many life-saving blood transfusions. The reversal of heart failure caused by accumulation of iron in the heart, by Desferal, was an important therapeutic advance. Moreover, the development of molecular biological techniques has now simplified prenatal diagnosis by allowing the sampling of fetal skin or placental cells. Nathan, who has contributed greatly to our knowledge of the thalassaemias, has made the case for science — more and more science — in our understanding and treatment of human disease immensely compelling. At the same time, the inspiring Samaritan role of a caring physician suffuses the book. This medical and scientific drama is so engagingly written that to open the pages will ensure its reading.

Throughout the book, Nathan refers

to many contemporary leaders in science and medicine, particularly those concerned with unravelling haematological disease. At times his enthusiastic admiration for his colleagues leads him to such hyperbole that those referred to may justifiably experience twinges of personal embarrassment.

There is no doubt that Nathan has written a remarkable book that demonstrates the compatibility, indeed necessity, of combining hard science, medical technology and human compassion. The peaks and valleys of emotion that accompany success and failure in the treatment of chronic disease have never been better described. His account highlights over and over again that clinical investigation, anchored firmly in molecular biology, will inevitably lead to better understanding of human disease. □

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Triumph in adversity

Hugh Freeman

An Anthropologist on Mars: Seven Paradoxical Tales. By Oliver Sacks. Knopf/Picador: 1995. Pp. 328. \$24, £15.99 (hbk); £6.99 (pbk).

Memory's Ghost: The Strange Tale of Mr. M and the Nature of Memory. by Philip J. Hilt. Simon and Schuster: 1995. Pp. 253, \$22.

WITH the progress of scientific medicine, the 'anecdotal case' has acquired an increasingly bad name, and it is particularly anathematized in the current movement for 'evidence-based' practice. Yet the very unusual patient has always had an essential role in getting doctors to shift their framework of thought about how both the body and the mind work. For the past 20 years, Oliver Sacks has demonstrated in book after book the enormous value that still attaches to that natural experiment involving some rare abnormality of the nervous system. These "seven paradoxical tales" show that the seam is far from exhausted.

If the cases have a fairly constant theme, it is the surprisingly nonmechanical plasticity of the nervous system in responding to changed needs. The artist who became totally colour-blind, for instance, at first found himself in an "alien, incoherent, and almost nightmarish world". Yet as time passed, his new landscape of greys took on a strange fascination and even beauty; he began to see "a world of pure form". His head

CAUGHT in the act: a worker ant *Sphecomyrma freyi* preserved in amber thought to be some 80 million years old. Taken from *Journey to the Ants: A Story of Scientific Exploration* by Bert Hölldobler and Edward O. Wilson, who, according to Deborah M. Gordon in a review of the book last year, "have done for ants what Leavis did for denim" (*Nature* 373, 292; 1994). Shortlisted for this year's Rhône-Poulenc Science Book Prize, the book is now issued in paperback by Harvard University Press at \$16.95, £10.50.



injury had removed the higher integration where perception of colour is fused with memories and associations to become meaningful. Yet Sacks believes it is beyond our present capacity to map "the subtle, higher-level neural consequences" of such a loss.

Tourette's syndrome and autism are both remarkable for being identified in practically every human society, little influenced by culture. The usually uncontrollable tics and bizarre behaviour of Tourette's, surprisingly enough, can be suppressed by absorption in a skilled task. They may even allow unusual levels of performance, as in the surgeon here who was quite normal when operating. Sacks proposes some form of subcortical epilepsy as the cause, with spontaneous stimulation of brain centres that are phylogenetically primitive.

Even more puzzling neurophysiologically is autism, illustrated here by two unusually gifted people. The 'savant' artist Stephen Wiltshire is already well known through his topographical drawings, but Sacks — who has spent much time with him — ends up largely baffled by what is missing in him as a person: "all our models...break down before him". Autism affects nearly one in a thousand people, half of these never use speech and 95 per cent lead very restricted lives. Even the few gifted ones show "a radically different, almost alien mode of mind and being". Their gifts, though, seem to support Howard Gardner's advocacy of separate forms of intelligence, each with its own algorithms. The autistic are impaired in social interaction, communication and imagination, lacking a normal ability to perceive their own and others' state of mind. In the end, Sacks regrets that proper understanding may demand both technical and conceptual powers that are for now beyond our capabilities.

One of his other cases was of a man who, through a massive brain tumour, lost all power to record new memory in the past 30 years. An even more dramatic example of this loss is the focus of Philip Hilt's examination of memory. He also writes for the informed layman, but less elegantly and more in the 'gee-whizz' school of journalism than in Sacks's *New Yorker* prose. It is, he admits, an unconventional mix of personal narrative and scientific reporting.

The subject (H.M.) had many of the midline structures of his brain removed in 1953 by a maverick neurosurgeon in Connecticut, in an attempt to treat his epilepsy that was bizarre even by the standards of the time. Since then, H.M. has become "the most studied patient in the history of medical literature", although this information has not become very widely known. He is unable to hold any memory for more than a few minutes, so that "each moment is a surprise".

Hilt has interviewed many of the leading researchers on memory, and gives a very useful summary of current developments. Like intelligence, memory has many components and it is now clear that these have different sites in the brain. The inputting process is a fragile one, memory is an act of construction rather than recording, and it is constantly reshaped over time. Both books show what can be learnt from unfortunate individuals who suffered "some limited but extraordinary damage to parts of themselves". Both authors acknowledge Gerald Edelman's view that brain structure is shaped by each person's experience, so that no model will make humans predictable. □

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Talking genes

Robert Foley

The Great Human Diasporas: The History of Diversity and Evolution. By Luigi Luca Cavalli-Sforza and Francesco Cavalli-Sforza. Addison-Wesley: 1995. Pp. 300. \$27.50, £21.95.

IN the good old days, palaeoanthropology was an exciting discipline. Each new hominid fossil discovered would overthrow the whole edifice of human evolution, and in an instant a million years could be added on here or an ancestor sidelined to extinction there. But in the past two years, discoveries of new species and new genera have resulted in no substantial change in the pattern of human evolution and hardly any blood on the table. Even the first Englishman is now legitimate.

Perhaps this is because genetics has become the pace-setter in studies of human evolution. But, in a way that is strikingly similar to the early days of palaeoanthropology, gene-based interpretations of human evolution are enormously unstable. Genes tell divergent stories of geographical patterns and the estimated age of ancestral populations. Resolution is more often along the lines of 'my gene is better than your gene' rather than integration, reminiscent of the best traditions of palaeoanthropological debate.

The stabilization of palaeoanthropology came about for three reasons. First, as the fossil record grew, each new discovery represented a smaller and smaller proportional increase in knowledge. Second, there has been an anchoring of the interpretation of hominid fossils to other branches of biology — evolutionary theory, primate and mammalian evolution, and genetics. And third, palaeoanthropologists began to ask a broader range of questions than just 'How old?' and 'Which is the true ancestor?'.

The same process can now be seen in human evolutionary genetics. More and more genes are being sequenced, certainly. There is a move to integrate genetic data with other aspects of human biology and to consider broader questions about processes at the level of populations. In all these developments, Luigi Luca Cavalli-Sforza has been the leading figure.

The Great Human Diasporas, written jointly with his son Francesco, is a popular summary of how this work developed and of modern knowledge of human genetic history. Conversational in tone, it works well as a critical and informed guide to human biological history and processes. Apparent detours into Italian surnames or the marriage practices of the Pygmies always come back to the central scientific issue: how can we use genetic

data to tell us about human history and contemporary human problems?

As the title suggests, the book is largely about the endless movements of human populations over millennia and the genetic traces left behind. But at another level, Cavalli-Sforza shows plainly the futility of trying to interpret genes without knowing so much more — about selection and drift, about processes of cultural transmission, about history and geography, about fossils, about anthropology, about statistics. It is an excellent introduction to the complexities of human genetic diversity, and should also be read by genome-dippers and those who talk confidently about race. As this book shows, human biological history is far too dynamic for monolithic racial categories to survive.

This is a gentle and modest book, lacking the hyperbole and over-wrought metaphors of much modern science writing. Its simplicity, though, is deceptive, for Cavalli-Sforza brings insight and subtlety to human evolutionary genetics. Besides, his last book was more than a thousand pages long and cost more than \$150, so this one is a bargain. □

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Universal Darwinism

David L. Hull

Without Miracles: Universal Selection Theory and the Second Darwinian Revolution. By Gary Cziko. MIT Press: 1995. Pp. 385. \$30, £25.50.

RICHARD Dawkins strikes again. He has inspired yet another investigator to take up his Darwinian cause, in this case to extend explanations in terms of variation and selection to all cases of adapted and adaptive complexity. Cziko has taken on a tall order, because such complexity manifests itself in everything from the vertebrate eye, the mammalian immune system and the central nervous system to mating behaviour in prairie chickens, religious rituals among Melanesians and scientific theories.

Cziko begins by explaining how standard Darwinian theory accounts for organic adaptations and rapidly proceeds to the more controversial cases, in particular human knowledge. For each area, Cziko shows how providential and instructional explanations always seem to precede and to appear more attractive than selectionist explanations. For example, one of the earliest explanations of organic

adaptations was literally God's divine plan. When biologists finally abandoned this extremely satisfying explanation, they tended to prefer such instructionist mechanisms as the inheritance of acquired characteristics to Darwin's explanation in terms of blind variation and natural selection.

For the immune system in mammals, the providential explanation is that each organism is born with all possible antibodies already formed. An instructional explanation is that antigens induce the body to produce the appropriate antibodies. As it turns out, the mammalian immune system behaves in a classical Darwinian fashion. Organisms produce massive numbers of diverse antibodies, only a few of which are ever used to attack invading foreign bodies. Similar explanations have also been suggested for the development of the central nervous system, but in this case no selectionist consensus has yet to emerge.

Once Cziko has established the credentials for selectionist explanations in biology, he proceeds to his main concern — learning, behaviour and cultural transmission. Here the opportunities for obfuscation lie in wait at every turn. Everyone seems to think that they know what they mean when they say that cultural evolution is 'Lamarckian', but Cziko presents some of the problems with this notion. For example, if a female dog teaching her young to hunt is a form of Lamarckian inheritance, then so is the transmission of fleas from the mother to her offspring. The trouble with treating cultural transmission as Lamarckian is that the distinction between Lamarckian and Darwinian inheritance turns on the genotype-phenotype distinction, a distinction that is far from apparent in cultural evolution.

The appropriate adjective to modify the term 'variation' is equally problematic. Darwin referred to variations "in all directions", that is, with no bias toward the variations that an organism might need. Such variations are certainly not 'chance' or 'random' if these terms imply lack of causation. Cziko opts for 'blind', but he is well aware that this adjective also begs to be misunderstood. In the context of perception, he asks whether it can make any sense to refer to vision as blind.

Scientists may be many things, but very few of them are blind. All Cziko intends in claiming that science is one more instance of blind variation and selective retention is that scientists are not prescient or clairvoyant, but this interpretation is too weak. As far as I know, no one has claimed that scientists, let alone genes and fruitflies, are clairvoyant. The issue is the effect that previous knowledge has on the process of discovery. Cziko acknowledges that it serves as a constraint. Is English really so impoverished that no word can accurately characterize the sort of variation that

occurs in selection processes?

Although Cziko is aware of the dangers of the terminology he has chosen, on occasion it gets him into trouble. For example, as an educational psychologist, he objects to providential education, but he also feels obliged to denigrate instruction. We all know the benefits of providing an environment in which students can make discoveries on their own, but this process just takes too long. Selection processes are wasteful and inefficient. Cziko agrees that the most successful teachers are those who find an optimal mix of instruction and freedom to innovate, but, in this discussion, technical and everyday uses of 'instruction' are, I'm afraid, being run together.

Early in his book, Cziko decries the sorry state of biological education in the United States, where 47 per cent of people polled in 1991 still believed in creationism. Later, in the context of cultural evolution, he asks if the traditional Balinese farmer is "in any way irrational and illogical in his adoption of centuries-old methods of rice cultivation?" The answer is no. Even though scientists think that the religious beliefs that inform rice farming in Bali are mistaken, the beliefs have stood these farmers in good stead over the centuries. Are first-world creationists any more irrational or illogical in continuing to believe in the miraculous creation of Earth 10,000 years ago? From the perspective of selectionist epistemology, the answer has to be no. Like it or not, false beliefs can be more highly adaptive than true ones.

Cziko outlines universal Darwinism as clearly and comprehensively as is possible in a book designed for a popular audience. Some readers will find his views as misleading as they are seductive. Others will find them highly suggestive, possibly worth pursuing in their own right. I find myself in this second group. But one problem remains for all sides. The evolutionary process of variation in all directions and selective perpetuation has produced an organism that finds it very difficult to understand this process. If understanding really is produced by something like a Darwinian process of variation and selective retention, then why are the general features of this process itself so difficult to understand? Evolutionary theory seems so easy that almost anyone can misunderstand it. □

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■ **Darwin's Dangerous Idea: Evolution and the Meanings of Life** by Daniel C. Dennett has just been published by Allen Lane in the United Kingdom at £25. For a review by Mark Ridley of the US edition, see *Nature* **375**, 457 (1995).

A developmental switch sufficient for flower initiation in diverse plants

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We have generated transgenic plants in which the flower-meristem-identity gene *LEAFY* of *Arabidopsis* is constitutively expressed. *LEAFY* is sufficient to determine floral fate in lateral shoot meristems of both *Arabidopsis* and the heterologous species aspen, with the consequence that flower development is induced precociously. Our results also suggest a new level of regulation during flower development, as indicated by the competence of the main shoot to respond to *LEAFY* activity.

FLOWERING in *Arabidopsis* is controlled by several intrinsic and extrinsic signals, including a developmental clock and day length¹. A primary response to floral inductive signals is the transcriptional activation of the flower-meristem-identity genes *LEAFY* (*LFY*), *APETALA1* (*API*) and *CAULIFLOWER* (*CAL*) at the shoot apex; these are required to impart a floral fate on newly organized assemblages of stem cells called meristems^{2–4}. When these genes are inactivated by mutations, meristems that would normally form flowers develop into shoots or shoot-like structures instead, which seems to be the default state^{4–10}.

Flower-meristem-identity genes have overlapping, and thus partly redundant, functions, and plants with mutations in single genes typically retain at least some flower-like structures^{4,9,11}. Because the degree to which flower-meristem-identity genes can substitute for one another changes during the life cycle and with different environmental conditions, it has been difficult to define precisely the roles of individual genes in determining floral fate. A further complication results from the cross-regulatory interactions among flower-meristem-identity genes. For example, RNA levels of *LFY* are much reduced in *apl cal* double mutants⁹, and the onset of *API* expression is delayed in *lfy* mutants (C. Gustafson-Brown and M. Yanofsky, personal communication).

To understand better the roles of individual flower-meristem-identity genes, we have analysed transgenic plants in which the primary flower-meristem-identity gene *LFY*^{4,6,12} is constitutively expressed throughout the plant. This approach overcomes several experimental limitations. First, transcription of *LFY* no longer depends on other meristem-identity genes or on factors that regulate floral induction. Second, by expressing *LFY* in shoot meristems, where other flower-meristem-identity genes are normally not active, we can assay the functions of *LFY* in isolation.

We demonstrate here that *LFY* encodes a developmental switch, the activity of which is sufficient to convert all lateral shoots into solitary flowers, so that flowers are induced precociously. The main shoot eventually also forms a terminal flower, but it is less affected than lateral shoots by constitutive *LFY* expression. Furthermore, the effects on the main shoot are modulated by day length, suggesting that meristems must acquire competence to respond to *LFY* activity. Finally, we found that constitutive expression of *LFY* has similar effects in other species, for example, the perennial tree aspen, which has a flowering time that can be reduced from years to months.

Conversion of *Arabidopsis* shoots

We generated transgenic *Arabidopsis* plants¹³ in which *LFY*⁴ expression is under the control of the 35S promoter from cauli-

flower mosaic virus. This promoter is active at high levels throughout all plant tissues¹⁴. In flowers, the 35S::*LFY* transgene provides at least as much *LFY* activity as the endogenous gene, as it can completely suppress the *lfy* mutant phenotype, when crossed into the background of the *lfy-6* null allele⁴ (not shown).

Most 35S::*LFY* transformant lines show a very similar, dominant and heritable phenotype. Secondary shoots that arise in lateral positions are consistently replaced by solitary flowers, and higher-order shoots are absent (Figs 1 and 2). Although the number of rosette leaves is unchanged from the wild type, 35S::*LFY* plants flower earlier than wild type, as the solitary flowers in the axils of the rosette leaves develop and open precociously. In addition, the primary shoot terminates with a flower. In the most extreme cases, a terminal flower is formed immediately above the rosette (Fig. 1b). This gain-of-function phenotype, the conversion of shoots into flowers, is the opposite of the *lfy* loss-of-function phenotype, the conversion of flowers into shoots^{4–6}. This demonstrates that *LFY* encodes a developmental switch that is both sufficient and necessary to convert shoot meristems into flower meristems.

The effects of constitutive *LFY* expression differ for primary and secondary shoot meristems. Secondary meristems are transformed into flower meristems apparently as soon as they develop, and they produce only a single, solitary flower. In contrast, the primary shoot meristem produces leaves and lateral flowers before being consumed in the formation of a terminal flower. These developmental differences suggest that a meristem has to acquire competence to respond to the activity of flower-meristem-identity genes such as *LFY*. One possible explanation for the differences between primary and secondary shoot meristems is that competence is established at the same time point throughout the whole plant, and that secondary meristems do not produce leaves or lateral flowers because their development is delayed relative to that of the primary meristem. However, solitary flowers in the axils of rosette leaves can develop long before the primary shoot is converted into a terminal flower, which is most evident in short-day grown 35S::*LFY* plants (see below). Alternatively, the activity of the 35S promoter might differ between primary and secondary shoot meristems.

Conversion of aspen shoots

Because constitutive expression of *LFY* can induce flowers precociously during the vegetative phase of *Arabidopsis*, we wondered whether this might be true for other species as well. We chose to study the effect of constitutive *LFY* expression in a perennial tree, hybrid aspen, which is derived from parental species that flower naturally only after 8–20 years¹⁵. 35S::*LFY* aspen plants were obtained by *Agrobacterium*-mediated trans-

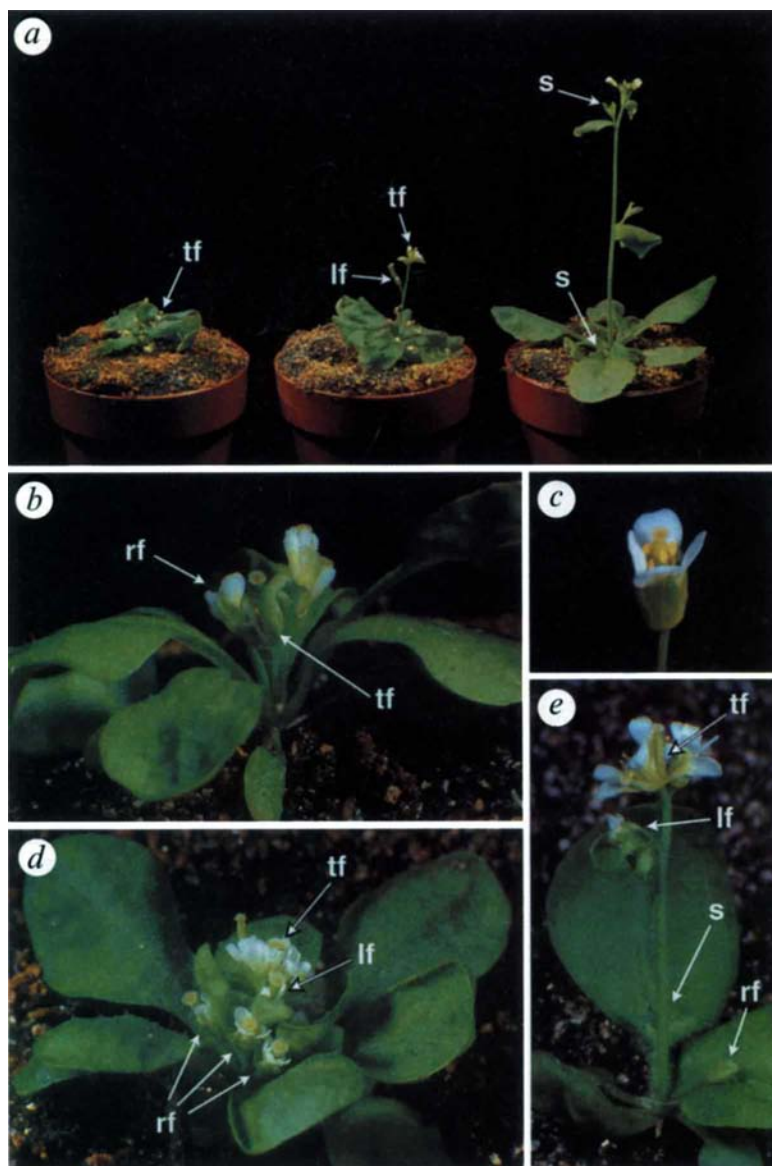


FIG. 1 *35S::LFY* causes transformation of *Arabidopsis* shoots into flowers. **a**, Four-week-old plants (ecotype Nossen) grown in long days. Left and middle, *35S::LFY* (line DW151.202); right, wild-type control. Development of the primary shoot has prematurely terminated in both *35S::LFY* plants. Some individuals, such as the one in the middle, develop a short primary shoot with a terminal flower (tf) as well as lateral flowers (lf) with bracts (see Fig. 4g), whereas others, such as the one on the left, do not produce an elongated primary shoot (see **b**). In wild type, complex secondary shoots (s) emerge from the axils of rosette leaves as well as from the axils of bracts on the upper part of the primary shoot, before flowers are initiated at the apex. **b**, Extreme *35S::LFY* phenotype (line DW151.202). After formation of a rosette, the primary shoot has immediately terminated with the formation of a solitary flower (tf), which in this case is somewhat abnormal. Additional solitary flowers (rf) have developed in the axils of rosette leaves. **c**, Close-up view of a lateral *35S::LFY* flower, with the normal complement of floral organs. **d**, **e**, Comparison of *35S::LFY* (line DW151.117) (**d**) and terminal flower (*tfl-2*) (**e**) phenotype in the *erecta* background; both panels are of same magnification. Although the primary shoot of the *35S::LFY* plant has produced a few lateral flowers (lf) in the axils of bracts above the rosette proper, no internode elongation has occurred, whereas substantial internode elongation is apparent in the *tfl* mutant. In the axils of rosette leaves of *tfl* mutants, either shoots (s) or solitary flowers (rf) may develop.

METHODS. A *LFY* complementary DNA⁴ was inserted into a T-DNA transformation vector containing a CaMV 35S promoter/3' nos cassette²³. Transformed seedlings were selected on kanamycin after *Agrobacterium*-mediated transformation, using the vacuum infiltration method¹³. Several hundred transformants in three different genetic backgrounds (Nossen, Wassilewskija and Columbia) were recovered, and several lines were characterized in detail. High levels of *LFY* RNA expression were detected by northern blot analysis. In general, Nossen lines tend to have weaker phenotypes, especially in short days (see Fig. 4). The *35S::LFY* transgene of line DW151.117 (ecotype Wassilewskija) was introgressed into the *erecta* background by backcrossing to a Landsberg *erecta* strain. Plants were grown under 16 h light:8 h dark.

formation of stem segments and subsequent regeneration of transgenic shoots in tissue culture¹⁶.

Regenerating *35S::LFY* aspen shoots initially produce solitary flowers in the axils of normal leaves (Fig. 3a, c). However, the number of vegetative leaves is limited, and the shoot meristem is prematurely consumed in the formation of an aberrant terminal flower (Fig. 3a). Precocious flower development is specific to *35S::LFY* transformants, as such an effect was not observed in non-transgenic controls (Fig. 3b). Furthermore, not a single instance of precocious flower development has been seen in the more than 1,500 other lines of transgenic aspen that were generated with various constructs during the past six years at the Swedish University of Agricultural Sciences^{16,17}.

Although wild-type *Arabidopsis* and aspen are rather different, one being a weed and the other a tree, the overall phenotype of *35S::LFY* aspen very much resembles that of *35S::LFY Arabidopsis*. In wild-type plants of both species, flowers are normally formed in lateral positions on inflorescence shoots. In aspen, these inflorescence shoots are called catkins and arise from the leaf axils of adult trees (Fig. 3d, e). In both *35S::LFY Arabidopsis* and *35S::LFY* aspen, solitary flowers form instead of shoots in the axils of vegetative leaves. Moreover, as in *Arabidopsis*, the secondary shoots of transgenic aspen are more severely affected than the primary shoot.

An apparent *LFY* orthologue from poplar has been described¹⁸ which, similarly to tobacco *LFY*¹⁹, is already expressed during the vegetative stage. The vegetative expression might have suggested that *LFY* activity is not sufficient to induce flower development in these species. Our results in aspen, which is in the same genus as poplar, indicate that this is not the case, a finding that extends to tobacco, which also flowers very early when transformed with a *35S::Arabidopsis LFY* construct (not shown). One possible explanation for the effects of *35S::LFY* in these species is that the transgene is expressed at higher effective levels than the endogenous gene. In tobacco, expression of the endogenous gene in the centre of the shoot meristem (which eventually turns into a flower meristem and forms a terminal flower) is relatively low¹⁹, and it is conceivable that other genes, such as *API*, are the primary regulators of flower-meristem identity in non-transgenic tobacco.

Effect of day length

To test whether the response to *LFY* activity is regulated by exogenous factors associated with floral induction, we examined the phenotype of *35S::LFY Arabidopsis* plants under short-day conditions. *Arabidopsis* is a facultative long-day plant, meaning that, although long days are not essential for flowering, they will greatly accelerate the transition to flowering¹. When plants of

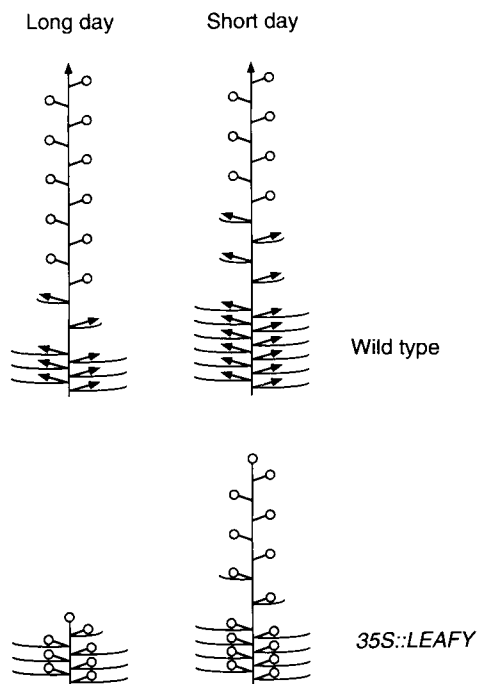


FIG. 2 Schematic comparison of wild-type and *35S::LFY* *Arabidopsis* plants. Arrowheads indicate shoot meristems, circles indicate flowers. In wild type, the primary shoot can be subdivided into a basal rosette, which contains leaves separated by short internodes, and an apical part with elongated internodes. The apical part, often referred to as an inflorescence, bears a few bracts (small stem leaves) with associated secondary shoots, as well as a potentially indeterminate number of flowers.

the ecotype Nossen are grown in short days instead of long days, flowering is delayed from 4 to 11 weeks, and the number of rosette leaves increases from 10 to 35. Short days also increase the number of rosette leaves produced by *35S::LFY* plants from 10 to 19, as well as the total number of nodes before the terminal

flower is formed from 14 to 49 (Figs 2 and 4a). Thus the effects of constitutive *LFY* activity are attenuated in short days.

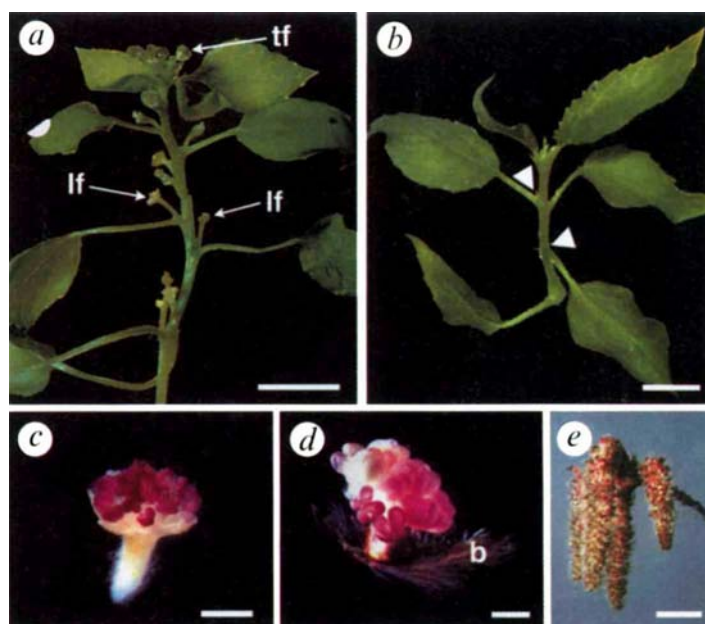
Surprisingly, the vegetative phase of the primary shoot is truncated, rather than compressed in short-day grown *35S::LFY* plants, which have fewer rosette leaves than wild-type controls. Wild-type rosette leaves that arise at different times have distinct characteristics, indicating that the vegetative phase has several distinct stages²⁰. One characteristic that changes during vegetative development is the presence and density of trichomes on the abaxial side (underside) of rosette leaves, and we used abaxial trichomes to monitor whether both early and late vegetative traits are expressed in short-day grown *35S::LFY* plants. In wild-type Columbia plants, abaxial trichomes appear at node 11–12, and increase in density on subsequent leaves (Fig. 4b–d). This timing is unchanged in *35S::LFY* plants, and abaxial trichomes appear at the same node as in wild type (Fig. 4e, f). Together with the reduced number of rosette leaves, this observation indicates a truncation of the vegetative phase, corroborating the view that *LFY* specifically and directly affects shoot meristems.

In contrast to the primary shoot meristem, secondary shoot meristems are much less affected by short days and, as in long-day grown plants, they are transformed into solitary flowers (Fig. 4a). However, that more leaves develop in short than in long days can also be interpreted as a slight attenuation of the effects of *LFY* on lateral meristems, if leaves and associated shoots (in this case, associated flowers) are viewed as a developmental unit. Such a view is supported by the observation that some of the more apical *35S::LFY* flowers bear leaf-like bracts on their pedicels (Fig. 4g).

Mediation of *LEAFY* activity by *APETALA1*

As well as *LFY*, mutations in the genes *API*, *CAL*, *APETALA2* (*AP2*) and *UNUSUAL FLORAL ORGANS* (*UFO*) are known to affect the identity of *Arabidopsis* flower meristems^{2,10,21,22}, although *lfy* mutations have generally the strongest effects, and are the only ones that consistently cause a complete transformation of at least a few flowers into shoots. Mutations in all five genes also affect the identity of floral organs, but meristem-identity and organ-identity defects are at least in some cases

FIG. 3 Constitutive expression of *Arabidopsis LFY* converts aspen shoots into flowers. a, b, Five-month-old shoots of hybrid aspen (*Populus tremula* × *tremuloides*) grown in tissue culture. a, *35S::LFY* transformant. Solitary, lateral flowers in the axils of leaves (lf) and an abnormal terminal flower (tf) are indicated. b, Non-transgenic control. Arrowheads indicate axils of leaves, from which lateral vegetative shoots will emerge, normally in the following year. Note that aspen plants regenerated from tissue culture show the same juvenile phenotype during the first growing cycle as plants grown from seed¹⁷. c, Close-up view of solitary male flower that formed in a leaf axil of a seven-month-old *35S::LFY* transformant that had been transferred to the greenhouse. d, Close-up view of male flower removed from wild-type catkin shown in e. Note bract (b) subtending wild-type flower. e, Cluster of male catkins of *P. tremula*, one of the parental species of hybrid aspen, taken from a 15-year-old tree. Red pigment in anthers is apparent (compare to c, d). Scale bars: a, b, 5 mm; c, d, 1 mm; e, 20 mm. METHODS. Hybrid aspen was transformed as described previously¹⁶. Levels of *LFY* RNA expression were similar to those of *35S::LFY Arabidopsis*, as determined by northern blot analysis. The number of vegetative leaves varied between different regenerating shoots. Those with a higher number of vegetative leaves formed roots, allowing for transfer to the greenhouse. Individual flowers were removed either from primary transformants that had been transferred to the greenhouse, or from catkins collected in spring 1995 at Carlskem (Umeå, Sweden) from a tree whose age was determined by counting the number of annual rings in a core extracted with an increment borer at 1.5 m above ground level. Flowers were fixed in formaldehyde/acetic acid/ethanol, and destained in ethanol before photography.



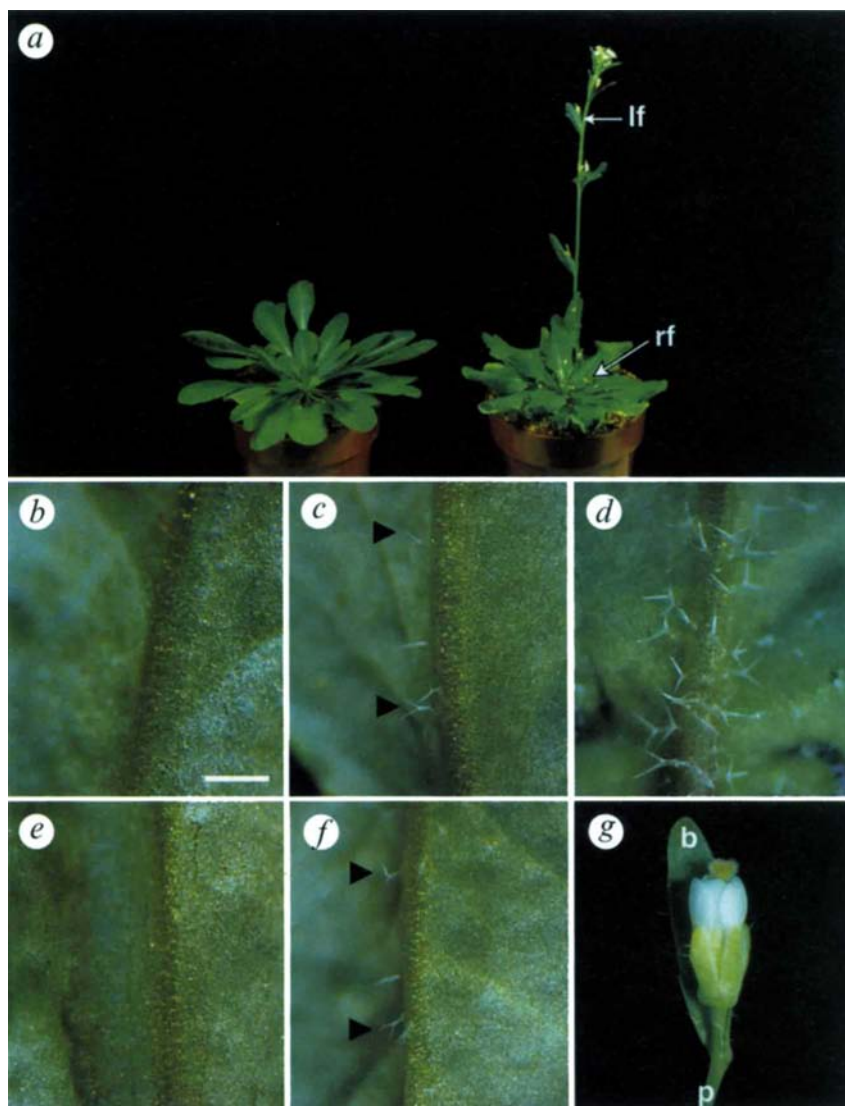


FIG. 4 *35S::LFY Arabidopsis* phenotype is attenuated in short days. **a**, Nine-week-old plants (ecotype Nossen) grown in short days. Left, wild-type control, which has no signs of flower development; right, *35S::LFY* plant (line DW151.202), which has twice as many rosette leaves as long-day grown siblings and many more flowers above the rosette (compare with Fig. 1a). Solitary flowers have formed both in the axils of rosette leaves (rf) and in the axils of bracts on the elongated part of the primary shoot (lf). **b–f**, Close-up views of proximal part of leaf midribs (abaxial sides), demonstrating trichome appearance. **b–d**, Control leaves (ecotype Columbia). **b**, Leaf 10, which is devoid of trichomes. **c**, Leaf 12, which in this individual is the second leaf with trichomes, which initially arise at low density (arrowheads). **d**, A typical late-phase rosette leaf (here, leaf 25), which has a dense covering of trichomes. **e**, **f**, *35S::LFY* transfor- mantants (ecotype Columbia). **e**, Leaf 10, which is the last rosette leaf of this individual (line DW151.1.4), and is devoid of trichomes, as the control shown in **b**. **f**, Leaf 12, which is the last rosette leaf of a different individual (line DW151.6.1) and has a low density of trichomes, similar to control shown in **c**. Scale bar for **b–f**, 1 mm (shown in **b**). **g**, Flower from the upper part of a shoot of a *35S::LFY* plant (line DW151.202). Note the bract (**b**) which inserts on the pedicel (**p**).

METHODS. Plants were grown in 10 h light:14 h dark.

separable^{9,23}. To determine whether any of the other genes are required to mediate the effects of *35S::LFY* on meristem identity, or whether their inactivation would merely affect the identity of floral organs in *35S::LFY* flowers, we crossed the *35S::LFY* transgene into various mutant backgrounds.

The *ap2-1*, *ap2-2* and *ufo-2* mutations cause only additive phenotypes, and do not significantly affect the shoot-to-flower conversion in *35S::LFY* plants. In contrast, the *ap1-1* mutation suppresses the *35S::LFY* phenotype to a notable extent, although terminal flowers are still formed (Fig. 5). Both the primary and secondary shoots are affected, with the strongest effects being observed in lateral positions (Fig. 5b, c). The solitary flowers that develop in the axils of rosette leaves of *35S::LFY API*⁺ plants (Fig. 1) become complex shoots with an average of 10 nodes in *35S::LFY ap1* plants (Fig. 5c). These observations not only confirm that *LFY* can induce *API* (which fails to become activated in early arising flowers of *lfy* mutants (C. Gustafson-Brown & M. Yanofsky, personal communication)), but also that the combined activities of *LFY* and *API* are more effective in transforming shoot meristems into flower meristems than *LFY* activity alone.

Taken together, these results suggest that competence to respond to flower-meristem-identity genes is acquired gradually. In young meristems, competence appears to be low, and both *LFY* and *API* are required to promote flower development over

that of shoots. Competence increases later in the life cycle, and *LFY* alone becomes sufficient to induce flower development.

Competence in floral induction

It has previously been recognized that a shoot apex has to acquire the competence to respond to floral inductive signals, such as those emanating from leaves upon changes in day length^{24,25}. In *Arabidopsis*, a primary response to floral induction appears to be the transcriptional activation of flower-meristem-identity genes in the flower meristems that form at the shoot apex^{2,4}. The simplest hypothesis would therefore be that floral competence is acquired through the ability of the promoters of these genes to be transcribed in response to inductive signals. However, our results indicate that floral induction regulates an additional, parallel pathway that affects the competence of meristems to respond to the activity of flower-meristem-identity genes (Fig. 6). A variety of possibilities for this second mechanism can be envisaged, among them that the response of floral target genes is regulated, and that these genes undergo a change from being completely repressed to partly derepressed. We favour such a hypothesis, because certain floral traits eventually develop even in *lfy ap1* double mutants^{4,6}, indicating that the requirement for *LFY* and *API* activity can be bypassed to a limited extent, and that (some) floral genes can become derepressed later in the life cycle of *Arabidopsis*. These effects are not due to residual activity

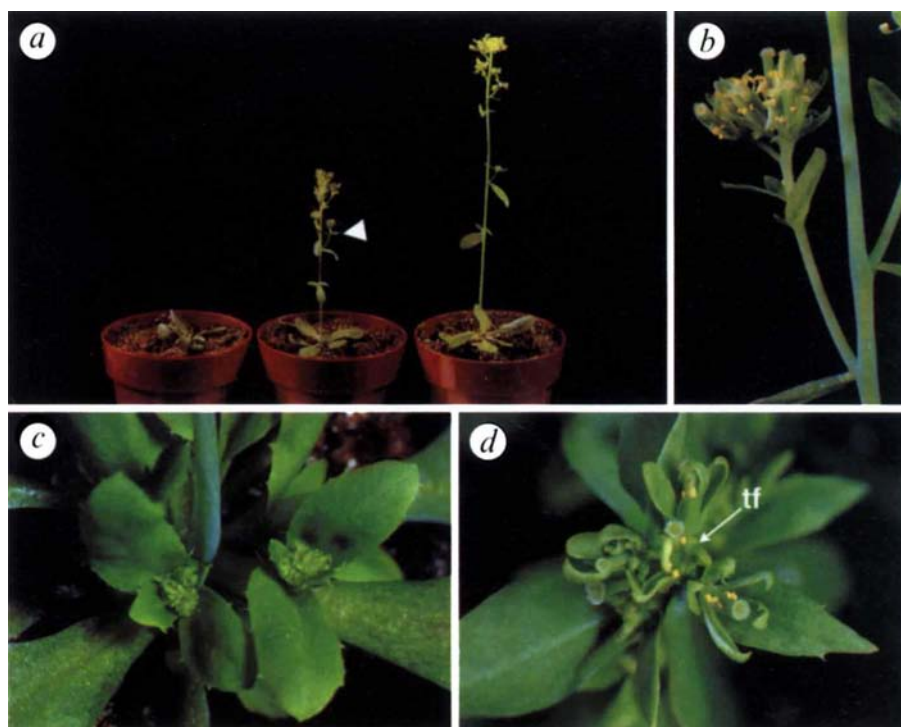


FIG. 5 *35S::LFY* phenotype is partly suppressed by an *ap1* mutation. **a**, Five-week-old plants that carry the *erecta* mutation. The *35S::LFY AP1*⁺ plant (left) has no elongated primary shoot. A primary shoot is well developed in the *35S::LFY ap1* plant (middle), although the primary shoot still terminates prematurely, and is shorter than that of the non-transgenic *ap1* plant (right). **b–d**, Detailed views of *35S::LFY ap1* plants. **b**, Close-up view of lateral shoot indicated by arrowhead in **a**. **c**, Emerging shoots in the axils of rosette leaves. **d**, Top view of primary shoot with terminal flower (tf). **c** and **d** are from a four-week-old plant. The *ap1* effects are enhanced further by the *cal-1* mutation, although there is no qualitative change in the *35S::LFY ap1* phenotype (not shown). **METHODS**. A *35S::LFY* transformant (line DW151.117, Wassilewskija ecotype) was crossed to *ap1-1* (Landberg *erecta* ecotype)¹⁰. Transheterozygote F₁ progeny was either backcrossed to *ap1-1* or allowed to self-fertilize. The *cal* genotype of selfed F₂ progeny was determined by polymerase chain reaction (PCR)³.

of other known flower-meristem-identity genes, as *cal*, *ap2* and *ufo* mutations do not enhance the *lfy ap1* phenotype⁹ (D.W., unpublished results; J. Levin & E. Meyerowitz, personal communication).

An important question is whether this second level of regulation is meaningful in non-transgenic wild-type plants. The ultimate test of our hypothesis depends on the isolation of mutations that specifically affect competence. Once such mutations have been identified, they can then be tested for their effects on non-transgenic plants.

We do not know at which point the two pathways, one leading to induction of flower-meristem-identity genes and the other regulating the competence to respond to flower-meristem-identity gene activity, diverge. These pathways must have at least some initial components in common, as they are both regulated by day length, the perception of which involves the red-light receptor phytochrome A^{26,27}.

LEAFY and TERMINAL FLOWER interactions

The *35S::LFY* phenotype is similar to that caused by mutations in the *Arabidopsis* gene *TERMINAL FLOWER* (*TFL*)^{28,29} (Fig. 1d, e). It has been suggested previously that *TFL* and *LFY* are antagonists^{7,8}, as *tfl* and *lfy* loss-of-function mutations have opposite phenotypes. Moreover, *TFL* is an important spatial regulator of *LFY*, such that *LFY* becomes ectopically expressed in *tfl* shoot meristems⁴. It is not yet known whether the reverse, that *LFY* is a spatial regulator of *TFL*, is also true. However, circumstantial evidence suggests that the antagonistic action of *TFL* and *LFY* is not merely due to mutual transcriptional repression, as the *tfl* mutant phenotype does not completely disappear even in a *lfy ap1* double-mutant background^{7,8}. This observation indicates that *TFL* might also be involved in the derepression of downstream floral genes (as discussed above) and, by extension, that *TFL* might control the competence of meristems to express floral genes in general. Still, *TFL* cannot be the only factor regulating the competence of meristems to respond to genes such as *LFY*, because *tfl* mutations do not eliminate the vegetative phase of the primary shoot meristem in *35S::LFY tfl* plants (not shown). Furthermore, although it is likely that at least some of the *35S::LFY* effects are due to reduced *TFL* activity, the interaction with *API* differentiates *tfl*

loss-of-function mutations and the *35S::LFY* gain-of-function allele, as *ap1* mutations attenuate the phenotype of *35S::LFY* plants but not *tfl* mutants²⁸. A more detailed investigation of the regulatory interactions between *LFY* and *TFL* must wait for the molecular cloning of *TFL*.

Perspectives

We have shown that constitutive expression of a single flower-meristem-identity gene, *LFY*, can induce precocious flower development in plants as diverse as *Arabidopsis*, an ephemeral weed, and aspen, a perennial tree. Our results not only contribute to the understanding of flower development and floral induction, they are also likely to be of interest because shorter flowering times lead to shorter generation times, which in turn allows acceleration of breeding programs. Modern crop varieties are the result of continued improvement by breeding, and two recently developed technologies have made breeding even more impor-

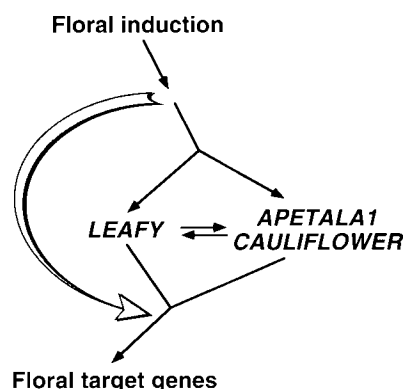


FIG. 6 Regulatory pathways of flower development. We propose that floral induction not only leads to transcriptional activation of flower-meristem-identity genes (black arrows), but that it is also involved in regulating the competence of floral target genes to respond to the activity of flower-meristem-identity genes (grey arrow). Flower meristem identity genes *LFY*, *AP1* and *CAL*^{2–4} positively regulate each other⁹ (C. Gustafson-Brown & M. Yanofsky, personal communication) and cooperate to activate floral target genes¹¹ (black arrows).

tant. The first technology is molecular mapping, with which genes encoding desirable traits can be rapidly located within the genome. Introduction of such traits into agriculturally important varieties is now greatly assisted by monitoring linked molecular markers, instead of testing for actual expression of these traits³⁰. The second technology is transformation of plants with hybrid genes conferring various traits such as engineered pathogen

resistance³¹. However, progress in this area has been delayed because the number of plant varieties amenable to transformation is often restricted, and extensive backcrossing is needed to introgress transgenes into a desired background. In both cases, marker-assisted breeding and transgene introgression, reduction of generation time through the induction of precocious flowering, should prove useful. □

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LETTERS TO NATURE

Observation of laser-induced fluorescent cooling of a solid

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THE possibility that an object might cool through its interaction with radiation was suggested as early as 1929 by Pringsheim¹. After Landau² established the basic thermodynamic consistency of such a process, certain aspects of fluorescent cooling were vigorously pursued^{3–11}. In particular, laser ‘Doppler’ cooling of gas-phase atoms and ions has today grown into a robust research area^{12–15}. In contrast, attempts to cool solids with light have met with limited success; non-radiative heating effects tend to dominate, and fluorescent cooling has at best resulted in a reduction in overall heating rates⁶. Here we report the experimental realization of net cooling of a solid with radiation. The cooling efficiencies achieved (up to 2%) are more than 10⁴ times those observed in Doppler cooling of gases. By pumping a fluorescent cooling element with a high-efficiency diode laser, it may be possible to construct a compact, solid-state optical cryocooler, thereby allowing widespread deployment of cryogenic electronics and detectors in space and elsewhere¹⁶.

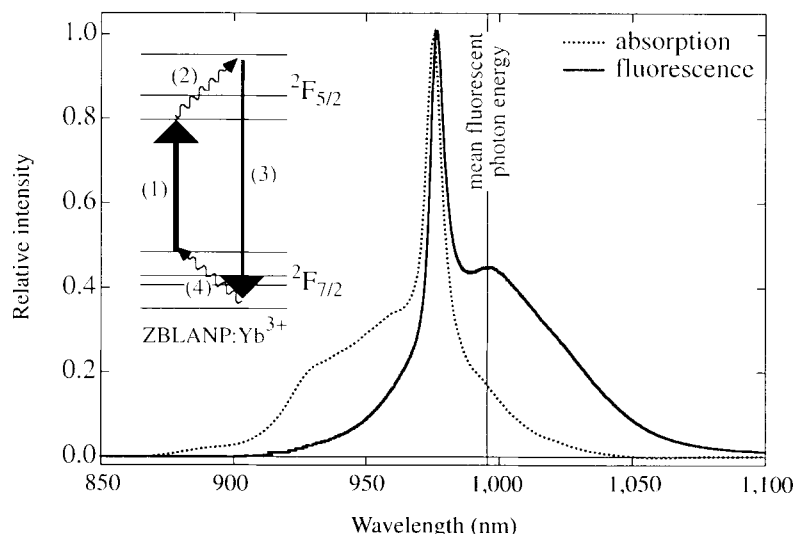
The basic principles and requirements of fluorescent cooling are illustrated by the processes occurring in our experiments which use a heavy-metal-fluoride glass (ZBLANP) doped with trivalent ytterbium ions. Each Yb³⁺ ion possesses only two groups of energy levels below the ultraviolet absorption edge of the host glass¹⁷. These groups are separated by an energy of

1.3 eV corresponding to a wavelength of ~1,000 nm. The ground-state group is split into four levels and the excited-state group is split into three levels, as shown schematically in Fig. 1 inset. The main part of Fig. 1 shows the measured absorption and fluorescence spectra of a sample of ZBLANP doped with 1 wt% Yb³⁺. The mean energy of the fluorescent photons corresponds to a wavelength $\lambda_F = 995$ nm, as indicated by the vertical line. Pumping this glass in the long-wavelength tail of the absorption spectrum moves excitations from the top of the ground-state group to the bottom of the excited-state group. The relative populations of the Stark levels within each group are thereby shifted slightly out of thermal equilibrium. That is, the populations of the lower levels in each group are larger than they would be in complete equilibrium. By absorbing thermal energy from the host material, thermal equilibrium within each group is restored. Radiative decays from the excited- to the ground-state groups produce photons that carry off the absorbed radiative and thermal energies.

Successful fluorescent cooling requires that a negligible fraction of the decays from the excited- to the ground-state groups occurs through non-radiative, heat-generating processes. Cooling is, at best, proportional to the energy spread of each group, whereas non-radiative decays generate heat in proportion to the much greater energy spacing between the groups. A high radiative quantum efficiency is thus required to ensure that non-radiative heating does not overwhelm the fluorescent cooling. Kastler³ suggested that rare-earth ions in transparent solids could be effective fluorescent coolers because of their large quantum efficiencies.

Each fluorescent photon carries off, on average, thermal energy equal to the difference between the pump-photon and the mean fluorescent-photon energies. In the ideal case where there are no non-radiative relaxations from the excited- to the ground-state groups, the cooling power, P_{cool} , is proportional both to the absorbed pump power, P_{abs} , and to the average difference in the photon energies of the pump and fluorescent radiation. In terms of the wavelength λ of the pump radiation, the cooling

FIG. 1 Main figure, absorption coefficient (dashed curve) and fluorescence spectrum (solid curve) at room temperature for ZBLANP (ZrF₄-BaF₂-LaF₃-AlF₃-NaF-PbF₂) doped with 1 wt% Yb³⁺. The actual peak absorption coefficient is 1.1 cm⁻¹ at 975 nm. The fluorescence spectrum was recorded using a 1-m spectrometer and an InGaAs detector in a 90° scattering geometry, and was corrected for the nonuniform responsivity of the system. The shape of the fluorescence spectrum is the same regardless of where one pumps in the Yb³⁺ absorption band. The wavelength corresponding to the mean fluorescent-photon energy, λ_F , is indicated by a vertical line. Inset, schematic energy-level structure of ZBLANP:Yb³⁺; the splittings within each group have been exaggerated for clarity. The arrows denote a typical cooling cycle: (1) laser pumping, (2) energy absorption from the thermal bath, (3) radiative decay, and (4) additional thermal-energy absorption.



power is

$$P_{\text{cool}}(\lambda) = P_{\text{abs}}(\lambda)(\lambda - \lambda_F)/\lambda_F \quad (1)$$

We used two different experimental arrangements to investigate the fluorescent cooling of ZBLANP:Yb³⁺. In the first of these, photothermal-deflection spectroscopy^{18,19} is employed to measure the local temperature gradients induced by the pump laser inside a sample. The pump beam from a continuous-wave titanium-sapphire laser is focused into the sample. A helium-neon laser probe beam, coaligned with and slightly displaced from the pump beam, passes through the sample in the opposite direction. Angular deflections of this probe beam, which are caused by thermally-induced refractive-index gradients in the pumped volume of the sample, are measured with a position-sensitive photodetector. An optical chopper placed in the path of the pump beam modulates the photothermal-deflection signal.

Two examples of raw photothermal-deflection data recorded on an averaging oscilloscope are shown in Fig. 2a. This panel presents deflection waveforms synchronous with the chopper for pump wavelengths of 980 and 1,010 nm, respectively 15 nm below and above λ_F . The unmistakable 180° phase difference between the two waveforms indicates that the positive temperature gradient observed at 980 nm becomes a negative temperature gradient at 1,010 nm. Evidently, the probed volume of the sample is *cooling* at the longer wavelength.

The results of a more systematic investigation of the photothermal-deflection spectra appear in Fig. 2b, where filled circles show the measured deflection amplitudes normalized with respect to the incident pump-laser power. A clear transition from the heating to the cooling regime is observed as the pump wavelength is tuned to values larger than λ_F , indicated by the vertical line.

In the second experimental arrangement, the equilibrium temperatures reached by a bulk 2.5 × 2.5 × 6.9 mm sample are measured when the sample is continuously pumped by a titanium-sapphire laser beam. The sample is positioned at the centre of a vacuum chamber and rests on two vertical thin glass slides that thermally insulate the sample from the chamber. The inner wall of the chamber is painted black to absorb stray pump radiation as well as the fluorescence emitted by the sample. The pump-laser beam is directed along the long axis of the sample. The dimensions of the sample transverse to the optical axis are small enough that self-absorption of the fluorescence is minimal. The temperature of the sample is monitored with a liquid-nitrogen-cooled InSb infrared camera which is sensitive in the 3–5-μm range but blind to the ~1-μm laser and fluorescence radiation. Because ZBLANP is transparent at wavelengths shorter than 5 μm, a 1-mm² square of gold foil painted black on its outer

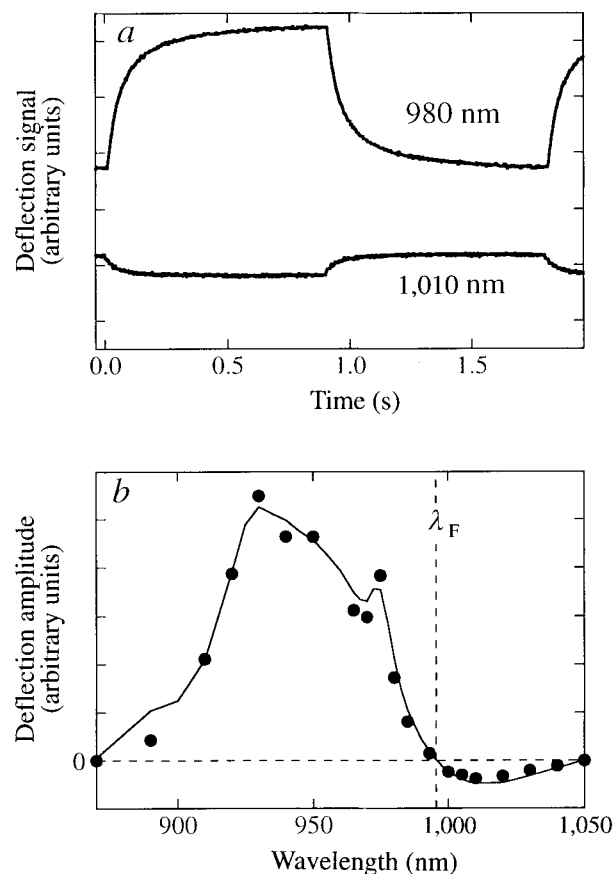


FIG. 2 *a*, Photothermal-deflection waveforms recorded on an averaging oscilloscope for pump-laser wavelengths of 980 and 1,010 nm, at a chopping period of 1.8 s. This period is long compared to the relevant atomic and thermal relaxation timescales of the sample. The corresponding laser powers are 1.03 and 0.70 W, respectively, with a focused beam diameter of ~50 μm. The sample length is 2.7 cm. The ~50-ms rise and fall times depend on the lateral separation between the pump and probe beams. *b*, Amplitudes (filled circles) of the photothermal-deflection waveforms normalized by the incident laser power as a function of pump-laser wavelength. The solid curve is a plot of equation (1), scaled by an arbitrary temperature-to-deflection conversion factor.

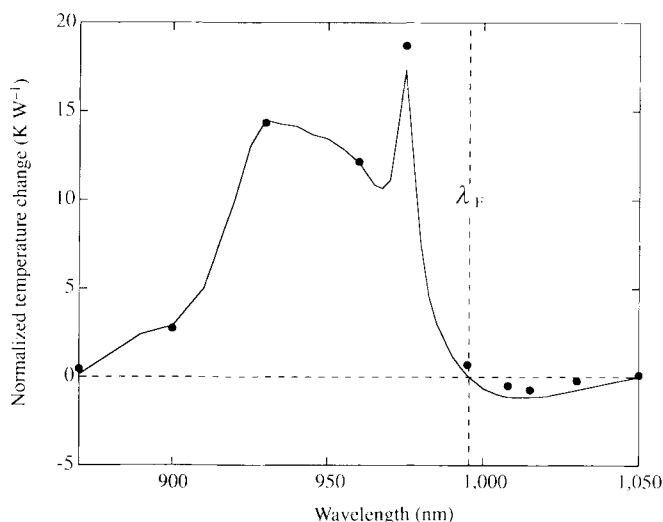


FIG. 3 Equilibrated temperature differences (filled circles) of a laser-pumped 0.69-cm-long bulk sample relative to an identical reference sample. Measurements are made with an infrared camera thermometrically calibrated by comparison to a type-T thermocouple. Temperature changes are normalized by the incident laser power. The values between 1,008 and 1,030 nm are less than zero, corresponding to cooling. The solid curve is a plot of the expected temperature changes from equation (2), reduced by 20% to allow for laser radiation that misses the sample. The differences in the data near the peak at 975 nm, compared to those of Fig. 2b, are due to differences in sample lengths and hence optical depths.

face is attached to the sample to enhance the emissivity at the detection wavelengths. The outer surface of the foil emits thermal radiation characteristic of the temperature of the sample, while the polished inner surface reflects at least 90% of the fluorescent radiation. To account for the changes in the sample temperature arising from temperature drifts of the chamber, a reference sample with an identical gold foil is positioned ~1 cm away from the test sample, outside the pump-beam path but on the same glass supports in the chamber. Temperature differences of 0.02 K between the pumped and reference samples can be

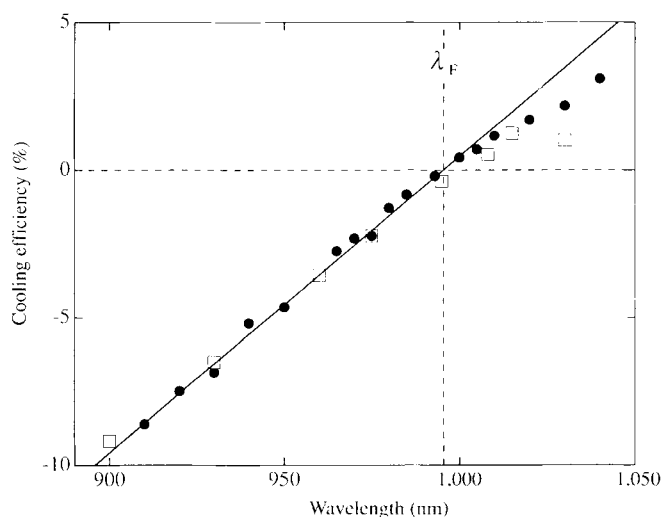


FIG. 4 Cooling efficiencies measured in the photothermal-deflection (filled circles) and bulk-cooling (open squares) experiments. Negative efficiencies correspond to heating. The amplitudes of the two data curves have been adjusted using the scaling factors from Figs 2b and 3. The solid curve is a plot of P_{cool}/P_{abs} from equation (1).

resolved. When the test sample is exposed to the pump laser, we find that the sample temperature equilibrates in about 15 min.

The filled circles in Fig. 3 show the steady-state temperatures of the test sample relative to the reference sample as a function of the pump wavelength per watt of incident laser power. Note that negative temperature differences as large as 0.3 K are obtained at long pump wavelengths. These results definitively demonstrate the absolute cooling of a solid by laser-induced anti-Stokes fluorescence.

The equilibrium temperature, T_s , of the sample is established by a balance between the laser-induced fluorescent cooling and the heat load from the environment. In our experiment, the dominant heat load is from the radiative coupling between the walls of the vacuum chamber at ambient temperature T_C and the sample. If the sample radiates as a black body, then

$$P_{cool} = \sigma A(T_C^4 - T_s^4) \quad (2)$$

where σ is the Stefan-Boltzmann constant and $A = 0.82 \text{ cm}^2$ is the surface area of the sample. For small temperature changes, equation (2) implies $\Delta T \equiv T_s - T_C \approx -P_{cool}/(4\sigma AT_C^3) \approx -2.0 \text{ K}$ ($P_{cool}/1 \text{ mW}$), with P_{cool} given by equation (1). This prediction is plotted as the solid curve in Fig. 3, scaled vertically by 0.8. The small discrepancies between theory and experiment in the cooling region can be reconciled if the radiative quantum efficiency equals ~0.997 and if ~0.1% of the laser power directly heats the sample. The results of the two types of experiments are in good agreement, as can be seen in Fig. 4, where the data from Figs 2b and 3 have been normalized by the corresponding absorptivities and plotted together for comparison. This normalization gives the cooling efficiency, defined as the ratio of the cooling power to the absorbed laser power. In agreement with equation (1), most of the data lie on a straight line. If the quantum efficiency were unity, the zero crossing would occur at $\lambda_F = 995 \text{ nm}$. Experimentally, the zero crossing agrees with this prediction to within 3 nm, indicating that the radiative quantum efficiency is at least 0.997. The deviations from linearity for $\lambda \geq 1,020 \text{ nm}$ may be explicable in terms of parasitic heating in our sample or experimental artefacts.

The experimental cooling efficiency in Fig. 4 is ~2% at a pump wavelength of 1,015 nm. Our computer model of the excitations and radiative transfer in the ZBLANP:Yb³⁺ system predicts that similar efficiencies should be achievable down to a temperature of 60 K. By pumping a fluorescent cooling material with an efficient diode laser, a fully solid-state cryocooler could be developed that operates with an efficiency comparable to commercial mechanical coolers¹⁶. This type of cryocooler would be functional in the temperature range useful for high- T_c superconductors, infrared detectors, and other cooled electronic devices and would be well-suited to space-based applications. □

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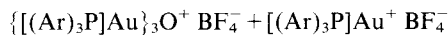
Synthesis of the gold analogue of the elusive doubly protonated water molecule

Hubert Schmidbaur, Stefan Hofreiter & Martin Paul

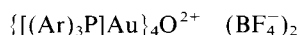
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SINGLY coordinated metal cations of the type $(L)M^+$ (where M is Cu, Ag or Au, and L is a donor ligand) are, in terms of their valence orbital characteristics, analogous to the proton¹. The analogy is particularly pronounced for the gold cation, in which relativistic effects strongly contract the 6s valence orbital², thereby permitting the incorporation of these proton analogues into stable molecular and ionic species having very short intramolecular bonds. Structural and stoichiometric parallels between gold cations and protons are illustrated by the species CH_4 and $C(AuL)_4$, NH_4^+ and $N(AuL)_4^+$ and OH_3^+ and $O(AuL)_3^+$ (refs 3–5), as well as by pairs of hypercoordinated cations such as CH_5^+ and $C(AuL)_5^+$ (refs 6, 7). We have recently synthesized the stable dicationic gold species $[(LAu)_6Cl]^{2+}$ and $[(LAu)_5N]^{2+}$ (refs 8, 9); their hydrogen counterparts, CH_6^{2+} and NH_5^{2+} , have never been observed and are expected to be intrinsically unstable¹⁰. Here we describe the preparation of the first stable four-coordinated dicationic oxygen compound, $[(LAu)_4O]^{2+}$, whose analogue, the doubly protonated water molecule $[H_4O]^{2+}$, has been predicted to be stable only in the gas phase, or to appear as a transient state during proton transfer in superacid media^{11,12}.

Treatment of tris $\{[tri(o\text{-tolyl})phosphine]gold(I)\}$ oxonium tetrafluoroborate (**1a**)⁵ with one equivalent of a freshly prepared solution of $[tri(o\text{-tolyl})phosphine]gold(I)$ tetrafluoroborate in a tetrahydrofuran/dichloromethane mixed solvent at -78°C gave a clear solution of the compound **2a**, which was isolated as a colourless solid in 95% yield on evaporation of the solvent in a vacuum (**2a** is the gold compound referred to in the title of this Letter). The triphenylphosphine homologue (**2b**) was prepared in 91% yield following the same procedure.



1a, b



2a, b

a: Ar = 2-CH₃-C₆H₄ (*o*-tolyl)

b: Ar = C₆H₅ (phenyl)

The products are thermally very stable, with decomposition temperatures as high as 202 °C (**2a**) and 117 °C (**2b**), and were readily identified by elemental analyses, NMR spectroscopy and field desorption mass spectrometry. The last technique provides direct and unequivocal evidence of the presence of the oxygen atom and the dicationic charge (see Box 1).

Single crystals of **2a** containing diethyl ether (Box 1) were obtained on cooling from dichloromethane/diethyl ether solutions. The cubic lattice (space group $Pn\bar{3}m$) contains dications

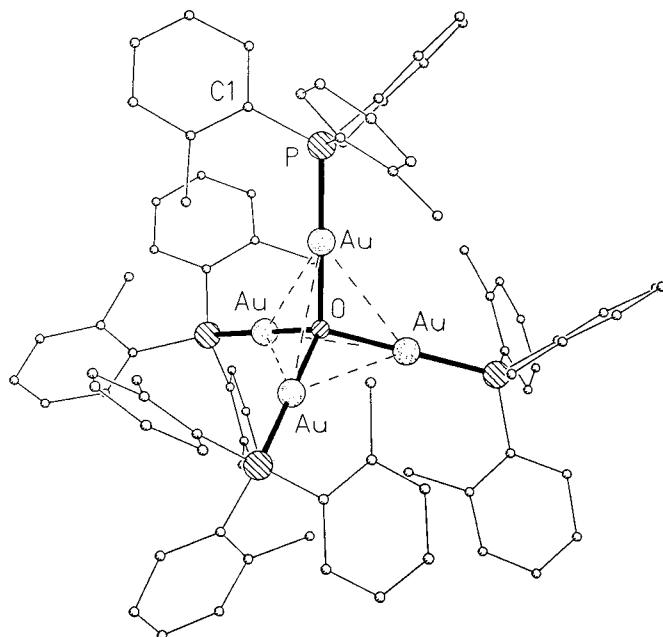


FIG. 1 Molecular structure of the dication $\{[(Ar_3P)Au]_4O\}^{2+}$ in crystals of the tetrafluoroborate salt (Ar is *o*-tolyl, **2a**). The anions are not shown; hydrogen atoms are omitted for clarity. Selected bond distances (Å) and angles (degrees): Au–O 2.0571(5), Au–P 2.225(4), P–C1 1.81(1), Au–Au 3.3593(8); Au–O–Au 109.5, O–Au–P 180.0, Au–P–C1 112.7(4).

and tetrafluoroborate anions. Imposed by crystal symmetry, the central oxygen atom of the dication is tetrahedrally coordinated by four gold atoms at a distance of 2.0570 (3) Å. Again by symmetry, the gold atoms are strictly linearly two-coordinate with Au–P distances of 2.225 (2) Å. The three *o*-tolyl groups at the phosphorus atoms form propellers of C₃ symmetry, which are directionally disordered (right- and left-handed propellers) in the crystal. In Fig. 1 only one of the rotational isomers is shown. As expected from symmetry criteria, the tetrafluoroborate anions are also disordered.

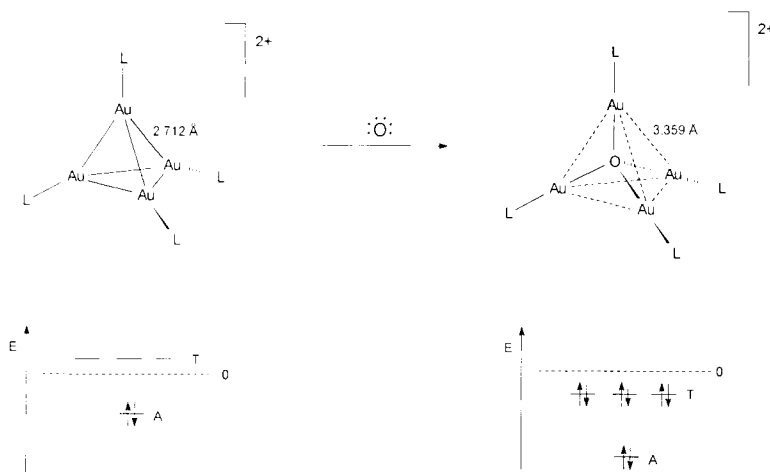
In dichloromethane-*d*₂ solutions at ambient temperature, each of the compounds **2a, b** shows only one resonance line in the

BOX 1 Analytical, spectroscopic and crystallographic data

COMPOUND **2a** C₈₄H₈₄Au₄B₂F₈OP₄ (*M*_r = 2,194.99): calcd C 45.97, H 3.86, Au 35.89; found C 45.04, H 4.16, Au 33.10. Mass spectrometry (field desorption): *m/z* = 1,010.6 (100%, $[(LAu)_4O]^{2+}$); 1,519.6 (10%, $[(LAu)_3O]^{+}$). $\{^1H\}^{31}P$ -NMR (CD₂Cl₂, 25 °C): δ = 2.7, s; -90°C : -8.1 , -8.8 , -9.3 (all s). 1H -NMR (CDCl₃, 25 °C): δ = 7.56 (t, *J* = 7.0, HC₄), 7.24 (m, HC_{3,5}), 6.84 (dd, *J* = 14.0 and 8.0, HC₆), 2.25 (s, Me). $\{^1H\}^{13}C$ -NMR (CDCl₃, 25 °C): δ = 22.6 (d, *J* = 10.0, Me), 122.3 (d, *J* = 64, C₁), 127.4 (d, *J* = 12, C₃), 132.4 (d, *J* = 9, C₅), 133.3 (s, C₄), 133.5 (d, *J* = 11, C₆), 141.8 (d, *J* = 9, C₂). Crystals of **2a** are cubic, space group $Pn\bar{3}m$ (no. 224 int. tables), *a* = 17.735 (1) Å, *V* = 5578.2 (5) Å³, ρ_{calc} = 1.307 g cm⁻³, *Z* = 2, *F*(000) = 2,108 e, μ (Mo-K α) = 53.65 cm⁻¹, *T* = -68°C , 2,618 reflections measured, 1,251 unique (*R*_{int} = 0.0185), 1,251 observed ($F_o^2 \geq -3\sigma F_o^2$), 86 refined parameters, *R* = 0.0357 ($F_o \geq 4\sigma F_o$), weighted *R*₂ = 0.1579, $\Delta\rho_{\text{min}}$ (max/min) +1.998/−0.641 e Å⁻³. Supplementary material has been deposited with Fachinformationszentrum Karlsruhe, D-76344 Eggenstein-Leopoldshafen, and may be obtained on quoting the full journal citation and the reference number CSD-59203.

Compound **2b** C₇₂H₆₀Au₄B₂F₈OP₄ (*M*_r = 2,026.69): calcd C 42.67, H 2.98, Au 38.88, F 7.50; found C 40.46, H 2.97, Au 37.35, F 7.01. MS (FD): *m/z* = 962.2 (100%, $[(LAu)_4O]^{2+}$), 1,393.3 (3%, $[(LAu)_3O]^{+}$), 721.1 (14%, AuL₂⁺). $\{^1H\}^{31}P$ -NMR (CD₂Cl₂, 25 °C): δ = 25.4, s; -90°C : 24.6, s. 1H -NMR (CD₂Cl₂, 25 °C): δ = 7.44–7.66 (m, Ph). $\{^1H\}^{13}C$ -NMR (CD₂Cl₂, 25 °C): δ = 127.0 (d, *J* = 67, C₁), 129.9 (d, *J* = 13, C_{3,5}), 133.3 (d, *J* = 3, C₄), 134.2 (d, *J* = 13, C_{2,6}).

FIG. 2 Top, the gold cluster dication $[(LAu)_4]^{2+}$ (L is PAr_3 , Ar is aryl) with and without an interstitial oxygen atom. Bottom, schematic drawing of the bonding molecular orbitals of the $[Au_4]^{2+}$ and $[Au_4O]^{2+}$ cores, respectively. (A and T represent fully symmetrical singly and triply degenerate orbitals.)



$\{^1H\}^{31}P$ -NMR spectrum, and one set of *o*-tolyl or phenyl resonances in the 1H - and ^{13}C -NMR spectra, respectively. This indicates the full equivalence of all four ligands and of their aryl groups. Any starting material $[(L)Au_3O]^+ BF_4^-$ remaining in solution is detected by separate resonances with distinctly different sets of NMR data⁵. These data are also clearly distinguished from the lines of the isoelectronic tetra(gold)ammonium salts $\{[(o\text{-tol})_3P]Au\}_4N^+ BF_4^-$ (**3a**) and $[(Ph_3P)Au]_4N^+ BF_4^-$ (**3b**) (ref. 4).

At very low temperature ($-90^\circ C$) the ^{31}P -NMR singlet lines of **2a** and **3a**, but not of **2b** and **3b**, are split reversibly into a closely spread set of signals. This splitting arises from the different relative orientation of the four individual tri(*o*-tolyl)phosphine propellers in each dication, which are not able to change rapidly their sign or rotation by concerted *o*-tolyl rotation (left-to right-handed) on the NMR timescale. Note that there is a set of isomers for **2a** and **3a**, which differ only in their individual combination of propeller directionality, but have non-equivalent phosphorus atoms. This is also the origin of the disorder in the cubic crystal (as discussed above). The hindrance of phenyl rotation in the Ph_3P groups of **2b**, **3b** is much less, and therefore no splitting is observed even at $-90^\circ C$.

Tetrahedral metallo-complexes of the oxide ion O^{2-} in an environment other than an oxide lattice are extremely rare, and the few examples reported in the literature are molecular (not dicationic) species stabilized by anionic ligand clamps or metal-metal bonds¹³. In the title compound the Au-Au distances (the edges of the Au_4O tetrahedron) are 3.3590 (4) Å long, which is still much shorter than the sum of the van der Waals radii of Au(I). It is therefore justified to ascribe the unprecedented stability of the $(LAu)_4O^{2+}$ unit to significant bonding between the closed-shell centres Au^+ ($[Xe]5d^{10}$, 'auriophilicity')¹⁴.

Note that the 'empty' tetrahedral dicationic $[(LAu)_4]^{2+}$ cluster has recently also been described¹⁵, and that it has much shorter Au-Au edges of only 2.712 Å (average), even though L represents the bulky *t*-Bu₃P ligand in this case. On insertion of an oxygen atom into this tetrahedron (Fig. 2), the six valence electrons of oxygen fill the three degenerate bonding orbitals obtained in point group T_d (or T) symmetry,¹⁶ which are vacant in the metal cluster Au_4^{2+} .

This model system is clearly important as gold metal does not form any stable binary oxide phase, but 'sub-surface oxides' are assumed to play an important role in heterogeneous catalysis at coinage-metal surfaces¹⁷. Silver metal in particular is widely used in the catalysis of oxidation processes, and convincing evidence has been accumulated that oxygen atoms thereby are not just adsorbed at the metal surface, but are actually entering lattice interstices between the first and second layers of silver atoms. These interstitial oxygen atoms are activating the surface silver atoms, the oxidation state of which is changed from zero in bulk metal to +1 at the surface. The bonding situation of an oxygen

atom in the new $[Au_4O]^{2+}$ units resembles that of a sub-surface oxygen atom in a tetrahedral void of a cubic close-packed lattice (typical of coinage metals). Attempts to isolate the silver analogue have not yet been successful. □

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Controlled reversal of lake acidification by treatment with phosphate fertilizer

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LAKES that have become acidified by airborne pollutants typically have low biological productivity and support an impoverished flora and fauna¹. In their natural state, these lakes were poorly buffered, and had a pre-industrial pH on the acid side of neutral². Although they can be neutralized by adding base, the resulting calcium-rich water supports plant and animal communities that are unlike those found in natural softwater lakes¹. It is known, however, that the long-term buffering of soft waters can be appreciably influenced by their biological productivity³⁻⁵. Here we report field-study results that show that by adding phosphate fertilizer to stimulate primary productivity, it is possible to generate sufficient base by

TABLE 1 Measured characteristics of Seathwaite tarn

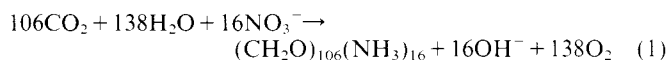
Variable	Units	Minimum	Mean	Maximum	Method
Max. depth	m	22.5	24.6	26	Lead line
Volume	m ³	2,399,910	2,906,996	3,256,571	Staff gauge
Area	m ²	207,503	250,402	262,309	Hypsographic curve
Rainfall	mm		2,486		Raingauge
Res. time	months		3		Calculation
pH*		5.01	5.16	5.32	Glass electrode
Alkalinity	µeq dm ⁻³	-30	-10.7	10	Gran titration
Total CO ₂ *	µeq dm ⁻³	11	17	28	GLC
Al	µM	3.6	6.5	9.3	Pyrocatechol violet
Ca	µM	23	28	33	AAS
Mg	µM	23	33	37	AAS
Na	µM	158	193	224	AAS
K	µM	7	9	13	AAS
SO ₄	µM	36	42	50	Ion chromatography
Cl	µM	191	228	269	Ion chromatography
NO ₃	µM	23	36	52	Ion chromatography
DOC*	mg dm ⁻³	0.3	1.2	2.9	Carla Erba
Chl a	µg dm ⁻³	0.3	1.2	1.9	Methanol/665 nm
DRP	µg dm ⁻³	<0.6			Molybdenum blue

The characteristics are shown along with mean and range values of determinands in all the integrated surface 5-m lakewater samples collected during 1991. (Abbreviations used: AAS atomic absorption spectroscopy, DOC dissolved organic carbon, Chl a chlorophyll a, GLC gas-liquid chromatography, DRP dissolved reactive phosphorus).

* Measured on single dip sample collected in sealed glass bottles.

the assimilation of nitrate to raise the pH of acid lake waters without drastically altering their community structure. Owing to the high efficiency of base production, only modest additions of phosphate are required, phytoplankton growth is not excessive and there is a marked increase in biological productivity at all trophic levels. In the longer term, additional quantities of base should be generated by the anoxic decomposition of organic material accumulating on the lake bed.

Previous studies⁶ concerned primarily with eutrophication have shown that base is generated when productivity is further stimulated by simultaneously adding phosphate and nitrate. When nitrate is assimilated it generates base (consumes acid) according to equation (1).



Base is also generated through the oxidative decomposition of organic material. If oxygen is the electron acceptor, there is no net generation of base, but if other inorganic oxidants such as nitrate, sulphate or iron oxyhydroxides are involved, their dissimilative reduction associated with the decomposition of organic matter generates base. The generated base is expressed as an alkalinity. It must not be confused with the temporary rise in pH associated with CO₂ consumption which affects neither alkalinity nor acidity. There is no advantage in adding nitrate rather than lime because 1 mol of nitrate is required to generate 1 mol of base. But providing nitrate is already present in the water, adding 1 mol of phosphate generates 16 mol of base through assimilation alone, making it remarkably efficient at base generation. As there are increasingly high concentrations of nitrate in many acid waters⁷, including most of those in Europe, adding modest amounts of phosphate may generate sufficient base to combat acidity without inducing excessive productivity. Here we describe a large-scale experiment which for the first time has been specifically designed to test the effect on acidity of adding phosphorus. Phosphorus is added to a lake to stimulate primary productivity and the natural base-producing mechanisms, including assimilation of nitrate, that are potentially almost self-sustaining.

The quantity of base generated by assimilative and dissimilative processes depends on many factors, including the flushing rate of the basin, the rate of incorporation into the sediment and gaseous transfers to the atmosphere⁵. In the initial stages of fertilization the only process of any significance is likely to be assimilative uptake of nitrate, but dissimilative processes will

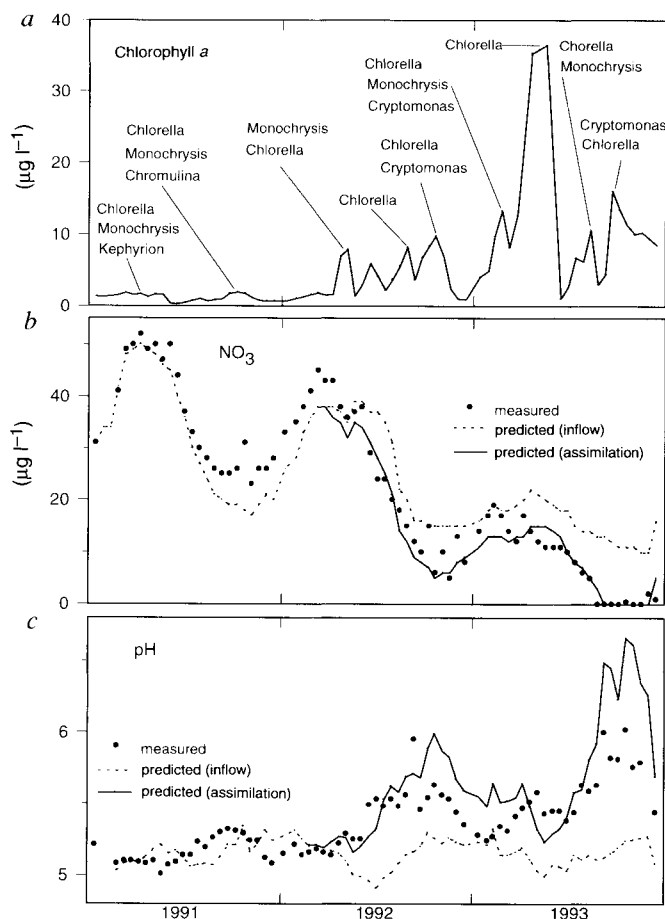
become increasingly important as the deep sediments become more anoxic.

We describe here the results of a fertilization experiment conducted in Seathwaite tarn, an upland reservoir in the English Lake District (54° 23' N; 3° 09' W). The results cover the first critical period of assimilative base generation when the quantity of base generated can be predicted from mass-balance calculations and the known additions of phosphate. Seathwaite tarn (Table 1) was chosen as the study site because it had a moderate acidity (pH 5), one main inflow (Tarn Head beck) and a gauged outflow. The experiment was conducted over a three year period; a pretreatment year followed by two years of fertilization. Dosing with phosphate commenced in March 1992 with the addition of a concentrated solution of sodium phosphate (50 g dm⁻³ of P) from a fast-moving boat. Approximately 75 dm³ was added every fortnight in 1992 and the dosing rate increased by ~50% in the summer of 1993. This treatment was estimated to have produced an average concentration of phosphate of 20 µg dm⁻³ in 1992 and 28 µg dm⁻³ in 1993. Water samples were collected at fortnightly intervals from the main inflow, the lake and the outflow. The inflow and outflow samples were collected using a flow-sensitive sampler which provides a reasonable approximation to flow-weighted mean concentration⁸. Throughout the experiment, the measured change in pH was compared with the pH predicted from a mass balance⁹ of the acidic species entering via the inflow (Fig. 1).

Figure 1a shows that most of the phosphorus added to the lake is rapidly assimilated by growing phytoplankton. The average concentration (µg l⁻¹) of phytoplankton chlorophyll in 1991 was 1.2, but this increased to 3.9 in 1992 and 12.7 in 1993. Most of this increase was due to the rapid growth of small species that are commonly referred to as 'nanoplankton'. These species are consumed in large numbers by filter-feeding crustacea which greatly accelerate the rate at which the fixed carbon is deposited in the deep sediment. Figure 1b shows the effect that the additions of phosphorus had on the assimilative uptake of nitrate in the lake. In 1991, the measured concentration of nitrate was very similar to that predicted by a mass balance of the nitrate entering the lake from the inflows and direct precipitation. In the growing seasons of 1992 and 1993, the measured concentration of nitrate was lower than that predicted by the mass-balance model, but was very similar to the concentration predicted by the assimilative-uptake model. Figure 1c shows the effect that this assimilative uptake of nitrate had on the pH of the lake. Before

FIG. 1 The effect of adding phosphate phosphorus to Seathwaite tarn in 1992 and 1993. *a*, The changes in the concentration of chlorophyll *a* with the dominant phytoplankton genera indicated. *b*, The change in the concentration of nitrate nitrogen. *c*, The change in pH. Two predicted plots for nitrate and pH have been included in *b* and *c*.

METHODS. The 'inflow' predictions use fortnightly measurements of concentrations in the inflow and rain in mass-balance calculations which assume a one-box model⁹ for the lake water. Outflows were calculated from the measured inflow adjusted by volume changes estimated from the measured lake level. Concentrations in the outflow were assumed to be the same as those measured in the surface 5 m of the lake. The inflow concentrations in the main stream accounting for 64% of the total inflow were assumed to be representative. Direct precipitation inputs onto the lake surface were almost negligible, but included. The 'assimilation' predictions further assume that nitrate is assimilated according to the Redfield ratio, that is 16 mol of N to each mole of P added. To predict pH an acidity function, $AF(\mu\text{eq dm}^{-3})$, was used in the mass-balance calculations. The carbonate and Al species were calculated from the measured p_{CO_2} and inorganic Al in the stream. Temperature, pH and DOC are the other necessary input parameters. $AF = [H^+] + [Al(OH)^{2+}] + 2[Al^{3+}] + [Al(OH)_3] - [Al(OH)_4^-] - [HCO_3^-] - 2[CO_3^{2-}] - [OH^-] + 3[DOC]$. E. Tipping (unpublished results) has observed that the acidity associated with DOC in acidic Lake District waters can be approximately represented in $\mu\text{eq dm}^{-3}$ by $3[DOC]$ where $[DOC]$ is in mg dm^{-3} . The resulting predicted value for AF in the lake was used, along with the measured concentrations of Al and p_{CO_2} to calculate the lake pH. The 'assimilation' predictions further assume that nitrogen is assimilated as nitrate in a 16:1 ratio to phosphorus, and that during this assimilation acid is consumed according to equation (1).



fertilization, the measured pH was similar to that predicted from the mass balance of inflow concentrations. After fertilization, the measured pH was consistently higher than that predicted by the simple mass balance. By assuming that base was generated during nitrate assimilation according to equation (1), a further model prediction of pH could be made. This 'assimilation' prediction is close to the measured pH in the early part of each year, but tends to be higher later in the year.

This work has demonstrated that addition of phosphate to an acidic unproductive lake can generate sufficient base through assimilation of nitrate to increase appreciably the pH. Moreover, the phytoplankton growth required to bring about this change was modest and did not give rise to any unacceptable changes in the qualitative composition of the community. Previous studies^{6,10} have used higher dose rates of fertilizer, resulting in eutrophic conditions inappropriate for treating acidity.

This preliminary work has only considered the generation of base by assimilation. It assumes that none of the organic material undergoes oxidative decomposition, a process which results in no net generation of base. For this first two years of treatment, most of the organic material is likely to be flushed from the lake or reside in the sediment, but some oxidative decomposition may contribute to the measured pH in the lake being slightly lower than predicted (Fig. 1c). In the longer term, a greater proportion of organic material is likely to decompose within the lake. However, if this occurs anoxically, further base will be generated by dissimilative reduction processes⁵. Such base generation has been shown to occur at the sediment-water interface of lakes with well oxygenated water columns³. If all the added phosphorus ultimately led to dissimilative reduction, ~100 mol of base could be generated from each mol of phosphorus added¹¹. Previous studies^{11,12} suggest that 32% of the assimilated organic material undergoes aerobic respiration, 25% is washed out of the lake,

25% is buried in the sediments, 13% is consumed by reduction of nitrate to nitrogen and 5% goes to FeS production and burial. According to the above assumption, 21 mol of base will be generated for each mol of phosphorus added to the lake. In Seathwaite tarn the mean pH would change from 5.16 to 5.85. As a progressively greater proportion of the photosynthesized organic material decomposes within the lake, more of the phosphorus will be released and be available to stimulate further phytoplankton growth. With time this recycling of phosphorus within the lake will increase the efficiency of the added phosphorus, ultimately providing sufficient positive feedback to approach a self-sustaining increase in productivity and pH.

This treatment will work best for lakes with a residence time in excess of a year. The poor enhancement of pH in Seathwaite tarn during high-flow winter months (Fig. 1c) is to be expected because its mean residence time is only 3 months (Table 1). Other attributes required for success are a modest initial acidity and a sustained loading of nitrate from the atmosphere or catchment. Because ammonia is preferentially assimilated in catchments it is rarely present in acid lakes. If ammonia was present, its assimilation would generate acid rather than base¹³, precluding treatment by adding phosphate.

Inducing such modest pH changes as the 0.5 pH observed here can be worthwhile. Solubility and adsorptive control of heavy-metal concentrations is particularly marked in the pH range 5–6, and so small pH increases may markedly reduce the metal content of polluted waters¹⁴. The poor productivity of most acid waters is associated with poor supplies of phosphorus. Fertilization of Seathwaite tarn has dramatically increased zooplankton and micro-benthic populations. Although it is too early to assess fully the effects on the scant brown-trout population, there are already signs of an improved growth rate.

It is operationally difficult to dissolve calcium carbonate whereas phosphate can be provided in a concentrated solution and easily added. For the treatment described here, a total of 5.9 m³ of phosphate fertilizer solution has been used. To bring about the same effect, 34 tonnes of calcium carbonate would have had to be added. Clearly the phosphate option can be cost-effective, especially if there is ultimately appreciable recycling of phosphorus. Most importantly, adding phosphate does not result in an unnatural chemistry and the pH is restored to pre-acidification levels rather than artificially high values.

Treating an acid lake with phosphate has the dual benefits of increasing the pH and productivity. Generally algal production is an important factor in controlling the long-term acidity of lakes. This study shows that phosphate can have beneficial effects within the environment, and that its complete elimination may enhance problems of low productivity and acidity. □

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Vertical mixing and coral death in the Red Sea following the eruption of Mount Pinatubo

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THE eruption of Mount Pinatubo in the Philippines led to a cold air-temperature anomaly throughout the Middle East during the winter of 1992¹. Here we report that the vertical mixing in the Gulf of Eilat (Aqaba) that winter was unusually deep—extending to >850 m—resulting in increased supply of nutrients to surface waters, which fuelled extraordinarily large algal and phytoplankton blooms. By spring, a thick mat of filamentous algae covered broad sections of the underlying reef causing extensive coral death. Branching colonies and solitary mushroom corals were most severely affected. This sequence of events, in which a short-term atmospheric cooling leads to a remarkable ecological response, is made possible by the unusually weak water-column stratification of the Gulf of Eilat. The depth of local vertical mixing during winter is determined by the net heat loss across the sea–air interface, so that anomalously cold winters drive the deeper mixing that can lead to increased phytoplankton blooms. Records of such events in fossil reefs may provide useful indicators of past variations in regional air temperatures.

The seasonal variability of air and sea-surface temperature in the Gulf of Eilat—a desert-bordered, 1,820-m-deep basin,

180 km long and 6–25 km wide—is shown in Fig. 1. As salinity varies little (40.3–40.8‰), vertical variations in water density are determined mainly by temperature². Evaporation in the Gulf (~350 cm yr⁻¹) greatly exceeds precipitation (~3 cm yr⁻¹)³. The compensatory influx of water to the Gulf, from the Red Sea, is confined by shallow sills to the upper warm layer³. Consequently, the temperature at depth is unusually high (for example, 20.6 °C at 1,200 m) and vertical stratification is weak^{2,4,5}.

The annual cycle of mixed-layer depth, seasonally driven by surface-water temperature changes, is shown in Fig. 2. As in oceanic regions, the deep vertical mixing is convectively driven by sea-surface cooling (that is, net upward heat flux), as opposed to mechanical wind mixing which usually maintains a shallow (<50 m) summer mixed layer. Interannual variations in the maximum depth of mixing (Fig. 3) are therefore directly linked to variations in the net heat flux during winter (November–March), with variations in the latter parameter accounting for nearly 88% of the variance in mixing depth (Table 1). That local sea-surface cooling controls the vertical mixing in the northern Gulf of Eilat, is further corroborated by a one-dimensional mixed-layer model, which successfully simulates the temporal variability and maximum depth of mixing (Fig. 3, Table 1). As the interannual variations in heat flux during winter are determined mostly by variations in latent- and sensible-heat losses (Fig. 3), and because these two sources of heat loss are strongly dependent on the air–sea temperature difference⁶, the latter parameter is highly correlated with the maximum depth of winter mixing (Table 1): variations in air temperature alone explain 81% of the variation in mixing. Winter averages of other parameters that could have potentially affected interannual variations in net heat flux, including solar radiation, relative humidity, wind speed and cloud cover, were not significantly ($P > 0.3$) correlated with either the net heat flux or the maximum mixing depth.

Seasonal changes in the biomass of phytoplankton (Fig. 1) and benthic algae⁷ follow the above mixing-stratification pattern. During summer, the stratified photic layer is depleted of nutrients and therefore supports low phytoplankton and algal biomass. As mixing reaches below the photic depth in late autumn (Fig. 2), nutrients are transported to the photic layer, enhancing phytoplankton and algal growth. The deep mixing during winter, however, although reaching nutrient-rich layers, does not allow further increase in phytoplankton density because of the transport of living cells to aphotic depths, where they respire but do not photosynthesize⁸. Spring blooms start when stratification starts, in mid-March (Fig. 1). As concentrations of nutrients increase with depth², more nutrients are transported to the photic zone during winters with deeper mixing. Thus, the correlation between the average surface chlorophyll concentration during the spring bloom (15 March–15 May) and the

TABLE 1 Correlation coefficients between variables

	Q_t	Air–sea temp. diff.	Mixing		Chlorophyll
			Observed	Model	
Q_t	—	—	—	—	—
Air–sea temp. diff.	-0.88*	—	—	—	—
Mixing (observed)	0.94*	-0.93*	—	—	—
Mixing (model)	0.98*	-0.85*	0.95*	—	—
Chlorophyll	0.96†	-0.86†	0.91*	0.97†	—

Pearson correlation coefficients between winter averages ($n = 7$ yr, 1988–95) of heat flux (Q_t), air–sea temperature difference, the maximum depth of observed and simulated mixing, and the spring-bloom average of surface chlorophyll a concentration (see Fig. 3 legend for details). The conventional sign of heat flux is positive upwards (sea surface cooling). Note that the 'observed' mixing depth for the winters 1988/89 and 1992/93 were underestimated because our profiles of salinity and temperature in these two winters did not reach the bottom of the mixed layer. Replacing the observed by simulated depths for those two years increases the correlation coefficients between the 'observed' values and chlorophyll concentration, air–sea temperature difference and heat flux by ~0.03 and improves the significance level for the latter two parameters to $P < 0.001$.

* $P < 0.01$, † $P < 0.001$, ‡ $P < 0.05$.

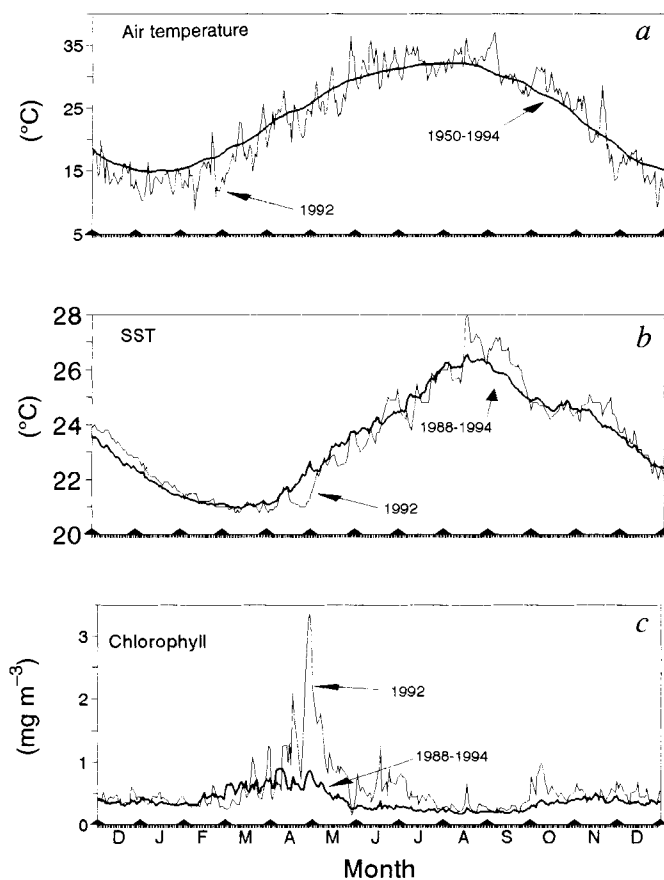


FIG. 1 Annual variations in: air temperature (a), sea surface temperature (b) and concentration of sea-surface chlorophyll *a* (c) in the northern Gulf of Eilat. Heavy lines, long-term averages; thin lines, daily values in 1992. Air-temperature measurements were made in Eilat since 1949 by the Israel Meteorological Service. Daily measurements of sea surface temperature and chlorophyll *a* were made since 1988 off the pier at the Coral World Observatory ~10 m seaward of the reef flat. Chlorophyll *a* was measured fluorometrically²⁴ after filtering duplicates of seawater samples on GF/F filters. Microscopic analyses of water samples taken at our site in 1991–93 (N. Kress, D. Lindell, B. Kimor, A. Neori and D. Angel, personal communication) showed that unidentified eukaryotic species comprised most of the phytoplankton biomass during the spring blooms.

net heat flux during the preceding winter is remarkably high (Table 1).

The highest net heat loss during the seven winters of our time series occurred in 1991/92 (Fig. 3). In fact, this winter was the coldest in the 46-yr record of air temperature in Eilat. By March, the vertical mixing exceeded 850 m (Fig. 2), unusually deep for a warm sea^{9,10}. Surface-water nutrient concentrations were also unusually high, with April (mid-bloom; Fig. 1) concentrations of dissolved nitrate at $1.2 \mu\text{mol l}^{-1}$, compared to a range of 0–0.4 $\mu\text{mol l}^{-1}$ during this month in normal years (B. Lazar and J. Erez, personal communication). The phytoplankton bloom was immense, lasting over two months; the usually clear blue waters of the Gulf became greenish, with concentration of chlorophyll *a* peaking at $>3 \text{ mg m}^{-3}$ (compared to typical concentrations of $\sim 0.7 \text{ mg m}^{-3}$; Fig. 1). An extensive algal mat (Fig. 4), dominated by the filamentous green alga *Enteromorpha* sp., covered the bottom from depths of 2 to over 25 m, along approximately the northern third of the Gulf. By the end of April, the mat was $>15 \text{ cm}$ thick ($>100 \text{ g dry wt m}^{-2}$), effectively shading corals and blocking their exposure to water flow. The blackened substrate under the mat and its strong odour, evident when pieces of the mat were brought ashore, indicated that decomposition of dead organic matter at the darkened and non-ventilated base of the mat caused anaerobic conditions rich with H_2S . This was an unusual phenomenon, as substrates at the coral reefs of Eilat are typically well ventilated and aerobic². Corals covered by the algal mat were severely damaged. Branching corals, mostly *Stylophora pistillata*, *Acropora* spp. and *Pocillopora verrucosa*, were most sensitive, with an average mortality rate of 20.5% ($\sigma = 15.7$), while $\sim 10\%$ of the surviving colonies suffered tissue damage spanning more than half of the colony surface (Table 2). Massive corals, mostly *Favia*, *Favites* and *Porites* spp. were apparently less sensitive, exhibiting an average damage of only 9.7% ($\sigma = 9.6$) of the colony surface. At a boulder field with high abundance of solitary mushroom corals (*Fungia granulosa*, *F. horrida* and *Ctenactis echinata*), located just north of the H. Steinitz Laboratory, nearly 71% of the 169 examined *Fungia* spp. were found dead. However, at a nearby reef patch where the algal cover remained sparse throughout the bloom period, the extent of mortality and tissue damage in branching corals was over an order of magnitude lower (Table 2), and only a single case of dead fungiid coral ($n = 52$) was observed. Based on limited observations, we suggest that the paucity of algae at the latter patch was due to localized high grazing pressure by herbivorous fish.

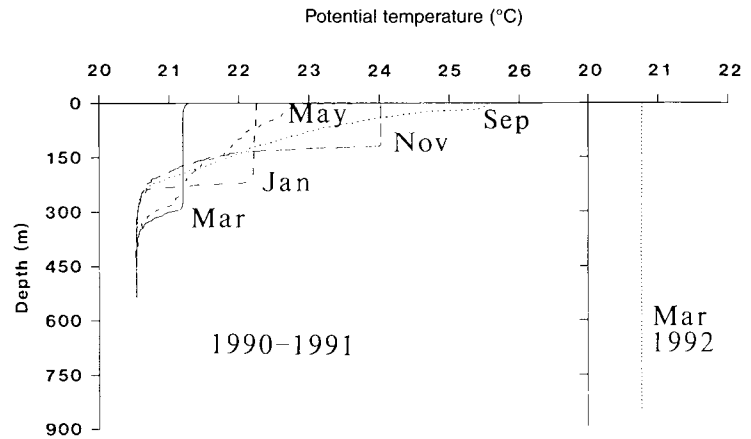
Possible causes for the mass coral death include the smothering of covered corals, the presence of hydrogen sulphide, some

TABLE 2 Coral damage after the algal bloom of spring 1992

Site	<i>n</i>	Branching species			Massive species		
		Average damage (% surface)	Proportion heavily damaged (%)	Proportion dead (%)	Average damage (% surface)	Proportion heavily damaged (%)	Proportion dead (%)
MBL	4	32.5 (18.4)	9.5 (3.8)	20.5 (15.7)	9.7 (9.6)	4.5 (5.3)	3.3 (5.0)
NR	3	3.8 (3.1)	0.8 (1.4)	0.8 (1.4)	1.1 (0.6)	0	0
<i>P</i> value		*	*	*	*	NS	NS

For each site (MBL and NR) is given the number of quadrats examined, the average extent of tissue damage, and the proportions of 'heavily damaged' and 'dead' colonies (defined as damage extending 51–94% and $\geq 95\%$ of the colony surface, respectively). Values in parentheses are standard deviations. Damage assessment was based on visual estimation of the proportion ($\pm 10\%$) of recently dead parts in each colony. Recent death was clearly distinguishable from older damage by the clean white colour and the intact texture of newly exposed skeleton. Sections damaged before the bloom ('old' damage) were eroded and encrusted with fouling organisms. The data were collected at the declining phase of the algal mat in late May 1992. The site MBL was located in front of the H. Steinitz Marine Biology Laboratory; NR was $\sim 250 \text{ m}$ to the north, at the southern section of the local Nature Reserve. The algal mat at MBL was fully developed, whereas algae at NR remained sparse throughout the bloom period. All corals $\geq 2 \text{ cm}$ in diameter were examined in three (NR) or four (MBL) $5 \times 5 \text{ m}$ haphazardly set quadrats at 5–7 m depth. A total of 216 and 109 branching, and 83 and 70 massive, corals were examined at MBL and NR, respectively. Significance values (*P*) are for intersite comparisons using the Mann–Whitney U-test (asterisk, $P < 0.05$; NS, not significant). The average extent of 'old' damage was small (3.5–5.8%) and was not significantly different between the two sites (Mann–Whitney, $P > 0.4$) for either branching or massive taxa.

FIG. 2 Vertical profiles of potential temperature near the northern end of the Gulf of Eilat. Left, the annual cycle for 1990–91, starting with a stratified column in September 1990, through a maximum mixing depth in March 1991, to the re-establishment of stratification in May 1991. The 1990/91 winter was relatively warm with a short sea-cooling period. Note the start of stratification near the surface in March. Right, the unusually deep mixing in March 1992, at the close of the anomalously cold winter (1991/92) that followed the Pinatubo eruption. Temperature measurements were made with a self-contained salinity, temperature and depth gauge (STD-12, Applied Microsystems, Canada). The STD was usually lowered to 400–600 m depth, except in March 1992 when the instrument was lowered to 850 m. Because the contribution of salinity to vertical variations in water density is negligible², vertical profiles of density and potential temperature are almost mirror-images of each other. That actual vertical mixing was taking place at times when the vertical profiles of potential temperature were straight is corroborated by the co-occurring homogeneity of chlorophyll *a* throughout the isothermal layer (down to 850 m in March 1992) (A.G., manuscript in preparation).

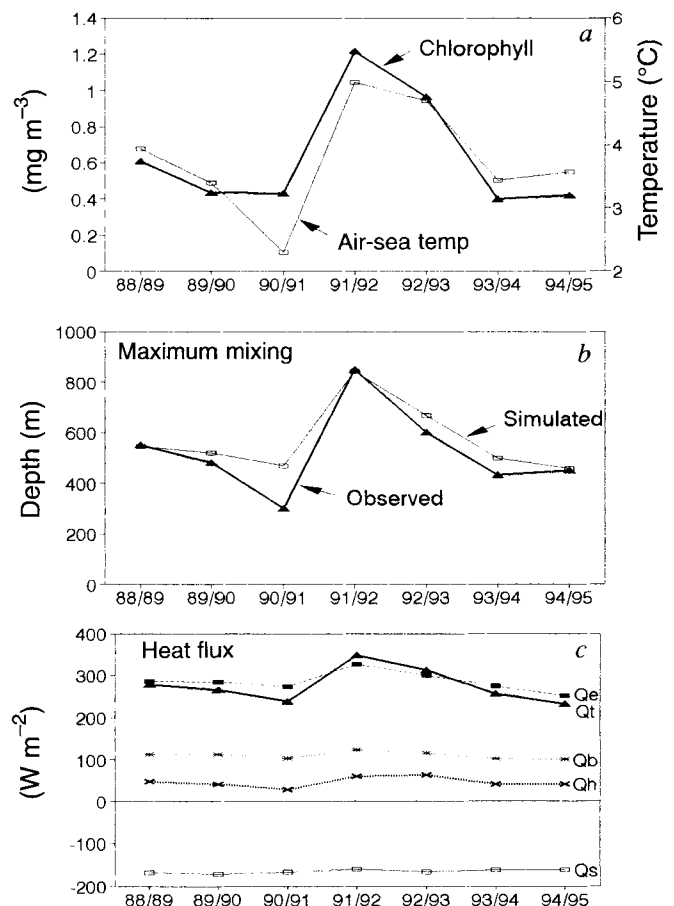


disease, or a synergistic interaction of factors, possibly including the phytoplankton bloom¹¹ and low ambient temperatures¹². Although the exact cause of the coral death is unknown, the lack of mass coral death where aquatic conditions (such as temperature, phytoplankton and nutrient concentrations) were virtually identical but the algal cover was sparse, indicates that the thick algal cover induced conditions necessary for the cause of mass death to become effective. Thus, the air-temperature anomaly that prompted the unusual algal growth during spring 1992 was ultimately responsible for the following mass coral death.

The anomalously cold Eilat winter of 1992 followed the explosive eruption of Mt Pinatubo¹ (June 1991). This eruption,

one of the largest in this century¹³, globally affected stratospheric circulation and surface temperatures^{1,14,18}. Records of air temperature and major volcanic events since 1883, examined by Robock and Mao¹ and supported by a general circulation model¹⁵, indicate that a significant cold anomaly occurs throughout the Middle East during the first winter after each major tropical volcanic eruption. Indeed, each of the five large eruptions listed by Robock and Mao¹ for the period of our air-temperature record (1950–94) was followed by a cold winter anomaly in Eilat. The second largest eruption (Mt El Chichon, Mexico, 1982) was followed by the second coldest winter in our record. A large algal bloom occurred also in spring 1983

FIG. 3 a–c, Interannual variations of biological, oceanographic and meteorological parameters in the northern Gulf of Eilat. a, Average concentration of surface chlorophyll *a* during the spring bloom (thick line) and the difference between air and sea surface temperatures during the preceding winter (thin line); b, the maximum depth of observed (thick line) and simulated (thin line) winter mixing (see below for simulation details); c, Winter averages of net heat flux (Q_t) across the sea–air interface and its four components: solar heating (Q_s), long-wave (infrared) back radiation (Q_b), latent (evaporative) heat loss (Q_e), and sensible (conductive) flux (Q_h). Winter is defined as the 151 days from 1 November to 31 March, encompassing the period of deep vertical mixing in the Gulf of Eilat; the spring bloom period is from 15 March to 15 May. The interannual coefficient of variation (CV) for the net heat flux was 15.2% ($n = 7$ yr). As indicated by the data in c, this CV value was mostly attributed to corresponding variations in latent and sensible heat fluxes (52 and 24%, respectively), with negligible contribution of the fairly constant solar radiation (<2%). Pairwise correlation coefficients between the presented environmental parameters and net heat flux are listed in Table 1. Computations of daily fluxes were based on measurements of solar radiation, wind speed, relative humidity, air temperature, sea surface temperature and cloud cover made at the monitoring station of the H. Steinitz Laboratory in Eilat. The annual values presented in the figure are averages of daily values. Berland's formula²⁵ was used to compute Q_s . Wind stress, Q_e and Q_h were computed using bulk aerodynamic formulae with stability-dependent exchange coefficient²⁶. To simulate the daily changes in mixing depths, a one-dimensional mixed-layer model based on the work of Mellor and Yamada^{27,28} was run for each winter, with values of surface fluxes and wind stress computed as above. Profiles of temperature and salinity typical for early November were used as initial conditions for each annual run. In agreement with observed data, the maximum simulated mixing always occurred in mid-to-late March and the deepest simulated mixing (842 m) occurred in March 1992. Note that in early 1989 and 1993, our STD profiler, although lowered to 550 and 600 m depth, respectively, did not reach the bottom of the mixed layer.



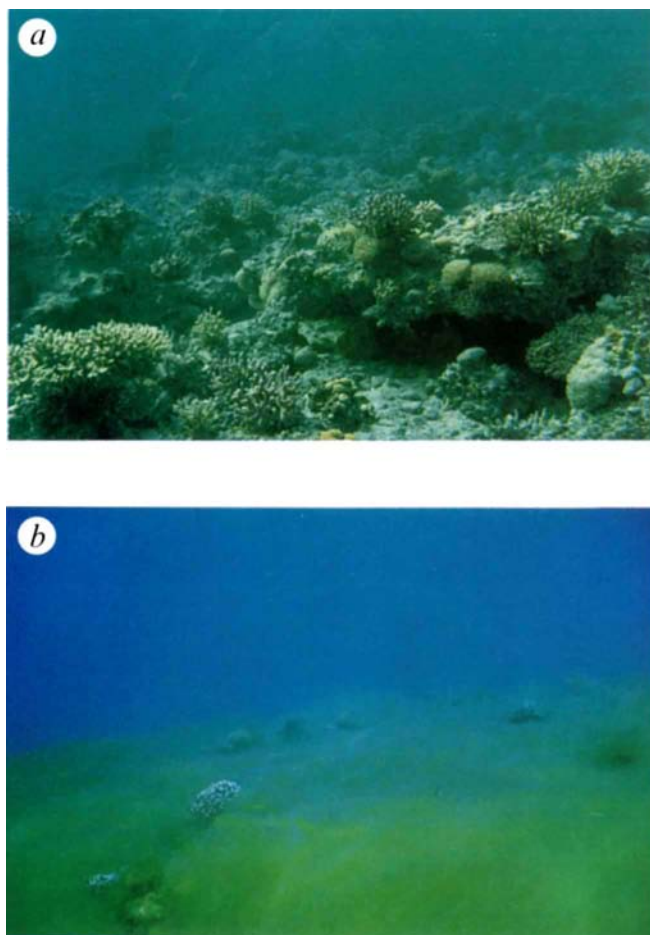


FIG. 4 The coral reef off the H. Steinitz Marine Biology Laboratory in Eilat at ~6 m depth. *a*, Under normal conditions (15 December 1994); *b*, at the peak of the algal bloom in spring 1992 (6 April 1992). The filamentous green alga is *Enteromorpha* sp. Corals growing on elevated substrates (seen in *b*) were not covered by algae and were therefore not damaged.

(A. Miroz, personal communication), but its effect on corals was unfortunately not examined. We note, however, that events of El Niño Southern Oscillation (ENSO) occurred in both 1983 and 1992 (ref. 19). Although several studies^{1,15} singled out volcanic eruptions as the dominant cause for these two cold winter anomalies in the Middle East, and indeed, in Eilat eight of the 15 ENSO events²⁰ during the past 45 years coincided with warmer-than-average winters, it is possible that the co-occurring ENSO events affected the magnitude of the anomalies^{20,21}.

The large algal bloom in spring 1992 was an ecological amplification of a rather short-term air-temperature anomaly. To the best of our knowledge, the weak vertical stratification that makes such amplification possible is unique to deep marginal seas with shallow sills.

Large volumes of water exchange heat with the atmosphere during the relatively short period of deep mixing. Therefore, sea surface temperatures in Eilat do not decrease much below 21 °C (average lowest daily sea surface temperature from 1988 to 1995 was 20.7 °C with a range of 0.7 °C, compared with 11.2 °C with a range of 4 °C for the minimum air temperatures). Palaeoclimate techniques using stable isotopes in marine sediments and corals^{2,22} would therefore be insensitive to such small interannual variations in local water temperatures. On the other hand, our findings indicate that records of massive eutrophication and spring blooms in the Gulf of Eilat, which are likely to be found

in fossil corals²³, may become useful indicators of interannual variations in air temperature. □

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A 20,000-year record of ocean circulation and climate change from the Santa Barbara basin

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MUCH of the evidence for climate-driven fluctuations in ocean circulation during the past 20,000 years has come from studies of the North Atlantic region^{1–6}. The extent to which such interactions have occurred in other ocean basins, and any associated teleconnections between basins, is poorly understood. Here we present high-resolution palaeoclimate and palaeoceanographic records from a 20,000-year sedimentary sequence from the Santa Barbara basin, on the eastern margin of the North Pacific Ocean. The sequence shows oscillations of the benthic environment between low-oxygen conditions (laminated sediments) during periods of warm climate, and higher-oxygen conditions (non-laminated, bioturbated sediments) during cool intervals. Age differences between coexisting benthic and planktonic foraminifers indicate climate-related changes in the age and source—and, hence, oxygen content—of basin bottom waters. Relatively young bottom waters are associated with the cooler intervals and are considered to reflect high proportions of intermediate waters derived from proximal sources. Conversely, older bottom waters are associated with the warmer

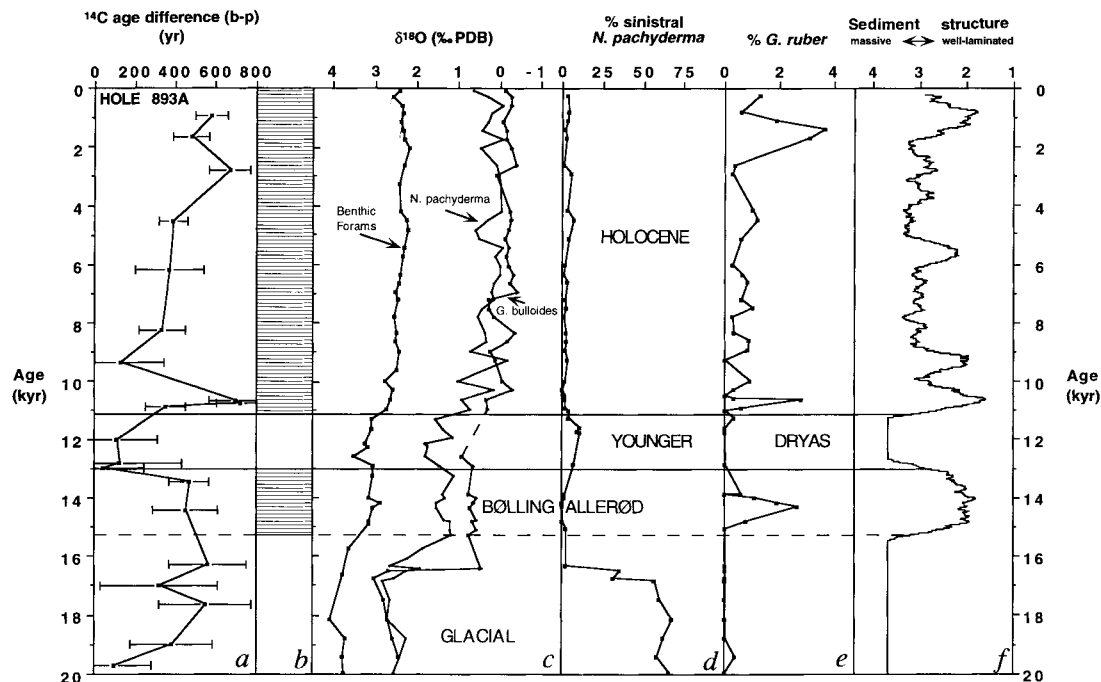


FIG. 1 Relations between inferred age of basinal waters (a), sediment structures (b, f) and palaeoclimate proxies (c, d, e) for the past 20 kyr (upper 32 m of Ocean Drilling Program Hole 893A, Santa Barbara basin, southern California). a, Benthic–planktonic (b–p) age differences (^{14}C) are considered to represent changes in age of basinal bottom waters¹². Reservoir-age correction used throughout is 825 yr (ref. 12); insufficient information exists to adjust for probable changes in reservoir ages with time. Chronology after refs 12 and 16. b, Presence (shaded) or absence (unshaded) of sediment laminations. f, Visual description of sediment structures at one-cm resolution for the entire sequence²⁶, grading from well-laminated to massive (1, well-laminated; 2, indistinctly laminated; 3, trace laminations; 4, massive or non-laminated). The curve in f was smoothed using a 99-cm running average to reduce variability and exhibit more clearly the major events addressed here. Smoothing has little effect on age of sediment facies transitions. These changes have been correlated to changes in degree of oxygenation and sediment

facies of Santa Barbara basin²⁶. Palaeoclimate proxies shown are: c, $\delta^{18}\text{O}$ records of the deeper-dwelling planktonic foraminifer *Neogloboquadrina pachyderma*, the shallower-dwelling form *Globigerina bulloides* and benthic foraminifers^{16,25}; d, e, percentage frequency changes in planktonic foraminifer assemblages (>150 μm) of the cool-water planktonic foraminifer, sinistral *N. pachyderma* (d) and the warm subtropical form, *Globigerinoides ruber*¹⁷ (e). No single benthic species occurs throughout the sequence due to large palaeoenvironmental changes¹⁶. We isotopically analysed *Bolivina argentea*, *B. spissa*, *B. tumida*, *Uvigerina peregrina curtica* and *Bulliminella tenuata*. We made interspecific oxygen-isotope comparisons and corrected *B. tumida* and *B. tenuata* by +0.25‰ relative to the other species¹⁶. Changes in palaeoclimate proxies²⁵ reveal the palaeoclimate episodes: Last Glacial Maximum, Bölling/Allerød interstadial, Younger Dryas cooling and Holocene warming.

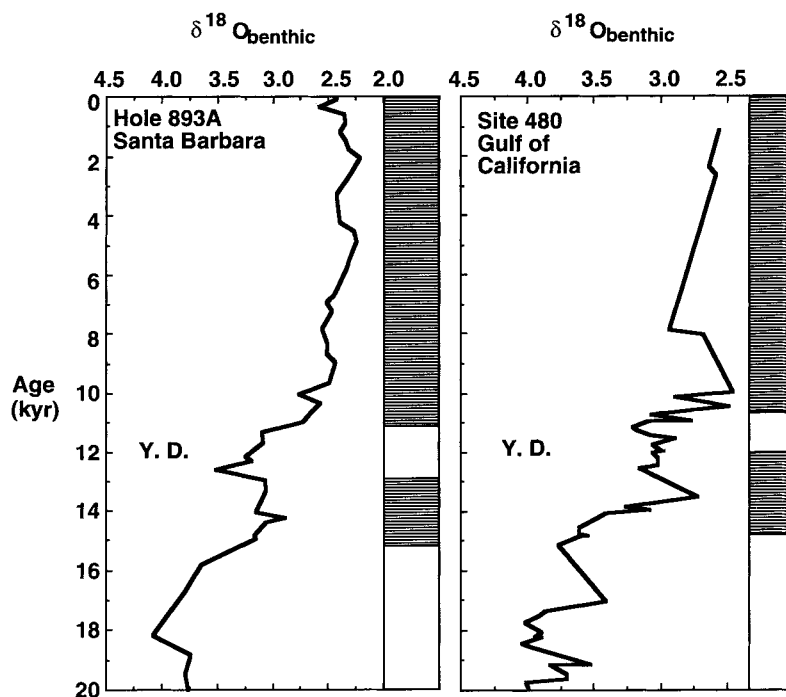
intervals and were derived from more distal sources. These climate-driven variations in ocean circulation appear to be synchronous with the main ocean–climate fluctuations in the North Atlantic region^{1–6}, suggesting that a tight coupling mechanism operates between the two basins.

The study site was continuously cored at 34° 17.25' N, 120° 2.2' W, 20 km south of the Santa Barbara coastline in a water depth of 588 m. We recovered a ~160-kyr, 196.5-m sequence of alternating laminated and homogenous (non-laminated) sediments⁸. This sequence is especially valuable for palaeoclimate analyses because of persistently high sedimentation rates (>120 cm kyr⁻¹) and near-continuous foraminifer assemblages abundant enough to generate high-quality stable-isotope, faunal census and radiocarbon age data. Santa Barbara basin is partially isolated within the California Borderland Province⁷. The present-day western sill (475 m) is at a depth of low oxygen concentrations (<0.8 ml l⁻¹) in the upper part of the oxygen-minimum zone⁹. Organic material derived from highly productive surface waters further reduces basin oxygen levels, and waters below ~500 m depth are anaerobic (<0.1 ml l⁻¹ oxygen), inhibiting bioturbation and preserving annual sediment laminations^{8,9}. In contrast, recent and Holocene sediments lack laminations at similar depths on the California Margin outside the Borderland Province^{10,11}. The past 20 kyr was dated using 32 accelerator mass spectrometry (AMS) radiocarbon ages of mixed planktonic foraminifera from 27 stratigraphic levels¹². We

calibrated radiocarbon ages to calendar years using two different methods^{13,15}, assuming a reservoir age of 825 yr (ref. 12). The samples range in age from 1,025 to 17,555 yr BP (reservoir-corrected) at intervals of $\leq 1,000$ yr, yielding¹² a near-linear sedimentation rate of 160 cm kyr⁻¹.

A total of 70 samples were taken for stable-isotope and faunal analyses at ~50-cm intervals in the upper 25 m (the last 16.6 kyr) and at ~100-cm intervals for the remaining core down to 32 m. Variations in $\delta^{18}\text{O}$ values of both planktonic and benthic foraminifers¹⁶ and planktonic foraminiferal census data¹⁷ exhibit climate oscillations characteristic of the Last Glacial Maximum and throughout the Holocene (Fig. 1). Four distinct climate episodes correlate with the well-known climate succession in northern Europe ranging from the Last Glacial Maximum (20–16 kyr) to the Bölling/Allerød interstadial (~15–13 kyr), to the Younger Dryas cooling (13–11.2 kyr), to the Holocene (<11.2 kyr). High $\delta^{18}\text{O}$ values mark the Last Glacial Maximum. Warming associated with termination 1A, the most recent deglacial episode, is diachronous between several proxies from 16.5 to 15 kyr. Initial warming is shown by a rapid switch at 16.5 kyr in planktonic foraminiferal faunas dominated by sinistral-coiled *Neogloboquadrina pachyderma* to warmer faunas with a high proportion of dextral-coiled *N. pachyderma*¹⁷ (Fig. 1). The coiling ratio of *N. pachyderma* also switched from >90% sinistral to <10% sinistral. This was immediately followed by a 1.75‰ decrease in $\delta^{18}\text{O}$ in the deeper-dwelling planktonic foraminifer

FIG. 2 Comparison of $\delta^{18}\text{O}$ records of benthic foraminifers and presence (shading) and absence (no shading) of sediment laminations between ODP Hole 893A (Santa Barbara basin) and DSDP Site 480 (Guaymas basin, Gulf of California)²⁹. Younger Dryas (YD) cooling is indicated by reversal towards increased $\delta^{18}\text{O}$ values in both sequences. Data plotted in calendar years. Calendar years for Site 480 calculated using a reservoir-age correction¹³ of 920 yr for original radiocarbon ages²⁹. Reservoir-age correction for Hole 893A is¹² 825 yr.



N. pachyderma. These changes indicate climate warming associated with northward migration of surface waters warmer than $\sim 10^\circ\text{C}$ across the site during termination 1A.

Relatively low $\delta^{18}\text{O}$ values between 15 and ~ 13 kyr record warmth correlated with the Bølling/Allerød interstadial. Further increases in warm-water planktonic foraminifers such as *Globigerinoides ruber* (Fig. 1), reflect increasing influence of the warm-subtropical countercurrent from the south. The Bølling/Allerød interstadial was succeeded by moderate cooling between 13 and 11.2 kyr that correlates closely with the Younger Dryas cooling episode of northern Europe¹⁸. In Hole 893A, Younger Dryas cooling is indicated by increases of $\delta^{18}\text{O}$ values in benthic foraminifers (0.5‰) and *N. pachyderma* (0.75‰), and increased relative abundances of cool-water planktonic foraminifers. Sinistral *N. pachyderma* exhibits a tenfold increase, and the coiling ratio of this species changed from ~ 98 to 80% dextral¹⁷. Full glacial conditions did not return during the Younger Dryas but are similar to those that mark the mid-point of termination 1A. Average sea surface temperatures remained above, but close to, 10°C .

We compare our ages of the Younger Dryas with those elsewhere using either a "reservoir-corrected" age (^{14}C age, 825 yr) and a calendar age (a calibrated ^{14}C age). In Hole 893A, the reservoir-corrected ^{14}C age for the beginning of the Younger Dryas is¹² 11,155 yr BP which is within 45–150 yr of radiocarbon ages for this event in North Atlantic sediment cores^{19,20} and New Zealand glacial deposits²¹. The calendar-year (calibrated) age of 12,970 BP at Santa Barbara basin¹² is remarkably close to an age of $12,940 \pm 260$ BP based on annual layer counts in the Greenland ice sheet^{22,23}. The reservoir-corrected (uncalibrated) age for the end of the Younger Dryas in Hole 893A is¹² 9,805 yr BP compared with the commonly accepted age²⁴ of $\sim 10,000$ radiocarbon years. The calibrated age is poorly constrained because of its occurrence during a 1,300-yr plateau on the calibration curve¹⁵ which occurred¹² between 10,900 and 12,300 yr BP. The calendar age for the end of the Younger Dryas in the Greenland ice sheet is^{22,23} $11,640 \pm 250$ yr BP. For Hole 893A, we use a calendar age of 11,200 yr BP based on interpolation between dated samples^{12,25}.

The end of Younger Dryas cooling is marked by decreased $\delta^{18}\text{O}$ values in benthic and planktonic foraminifers. Oxygen-isotope values in benthic foraminifers and *N. pachyderma* decreased during the early Holocene until ~ 6 kyr (Fig. 1) in

contrast to *Globigerina bulloides* which exhibits similar, although fluctuating Holocene $\delta^{18}\text{O}$ values after 10.3 kyr. The reappearance of warm-water planktonic foraminifers marks the end of the Younger Dryas (Fig. 1). Warm subtropical forms persisted during the remainder of the Holocene¹⁷, and indicating continuous influence of a poleward-flowing, warm countercurrent⁷. Throughout the Holocene (Fig. 1), climate fluctuated but was always warmer than the Younger Dryas^{17,25}. Basinal sediment facies is closely associated with climate change (Fig. 1), and the presence, absence and degree of biological disruption of laminations is controlled by changes in oxygen concentration of bottom waters^{8,26}. Laminations remain preserved when oxygen concentrations fall below $0.1\text{--}0.2\text{ ml l}^{-1}$ (ref. 27). The degree of preservation of laminations is inferred to be related to small changes in bottom-water oxygen concentrations and different degrees (millimetre-scale) of meiofaunal bioturbation²⁶, with non-laminated (massive) sediments indicating complete bioturbation beneath oxygenated waters. Benthic foraminiferal assemblages in laminated sediments are dominated by taxa typical of low-oxygen environments (*Bolivina* $\leq 90\%$; *Suggrunda* present). Non-laminated sediments contain a more diverse assemblage associated with well-oxygenated bottom waters²⁵. Assemblages from the massive Last Glacial Maximum sediments are usually dominated by *Epistominella* with *Nonionellina*, *Nonionella* and *Cassidulina*. During the Younger Dryas (non-laminated), the diversity of benthic foraminifers is intermediate between assemblages of the glacial maximum and the Holocene. Faunas are dominated by *Epistominella*, and *Uvigerina* is a consistent and sometimes dominant element. During non-laminated intervals, the low-oxygen-tolerant forms *Bolivina* and *Suggrunda* are absent-to-rare and echinoid spines (spatangids) are present. These spines are absent in laminated sediments. In the modern basin, echinoid spines are only present beneath waters with oxygen concentrations of $\geq 0.3\text{ ml l}^{-1}$ (interpreted from ref. 27).

Hole 893A records four major oscillations between laminated and non-laminated sediments during the past 20 kyr that have been semi-quantitatively described at one-cm resolution²⁶ (Fig. 1). The switch to laminated sediments at 15.2 kyr is closely associated with the beginning of the Bølling/Allerød interstadial (Fig. 1). Likewise, the climate changes at the beginning and end of the Younger Dryas are almost synchronous with the switches between laminated and non-laminated sediments (Fig. 1). The

stratigraphic resolution of our data is such that if any lag exists between climate and ventilation change, it would be ≤ 200 yr (Fig. 1). The Bølling/Allerød interstadial is marked by consistently strong laminations; in contrast, the Holocene sediments oscillate between weak and strong laminations²⁶ (Fig. 1). Our data is thus consistent with the hypothesis of synchronous to near-synchronous changes between global climate and ventilation in Santa Barbara basin.

The Santa Barbara basin sequence is not merely a record of local climate and oceanographic change, neither was it primarily controlled by local bathymetric configuration. Changes in basin ventilation are unlikely to have resulted directly from sea-level change because Younger Dryas cooling was associated with a pause rather than a fall in sea level²⁸. Furthermore, ventilation changes in Santa Barbara basin do not primarily record varying geographical or bathymetric isolation from the productive California Current because the facies changes during the Younger Dryas occurred without significant eustatic sea-level-controlled changes in basin configuration. Similar oxygenation-related facies changes are also recorded in DSDP Site 480 in Guaymas basin, Gulf of California²⁹ (Fig. 2) suggesting widespread changes in more isolated northeastern Pacific margin basins during the past 20 kyr. DSDP Site 480 (655 m water depth), on the slope of Guaymas basin, is not isolated by a sill at shallow depths and hence was not affected by changes in sea level²⁸. Poor ventilation in Santa Barbara basin seems to result from a delicate balance between warm deep waters, sufficiently high plankton productivity and relatively low oxygen concentrations of upper intermediate waters spilling into the basin from the Pacific. No consistent evidence exists from micropalaeontological^{17,30} and organic-carbon accumulation rate^{31,32} data for higher productivity during deposition of laminated versus non-laminated intervals. Furthermore, the benthic $\delta^{18}\text{O}$ record (Fig. 1) indicates the absence of any simple relations between changes in bottom-water temperatures and oxygenation in the basin. We believe that the cycles of oxygenation/dysaerobia in the basin were largely controlled by changes in oxygen concentrations of upper intermediate waters entering the basin, as has been suggested for the Gulf of California²⁹. Any restriction in circulation enhances small palaeo-oxygen changes to a greater degree than at locations on open continental margin slopes^{10,11}. Thus we suggest that relatively small changes in dissolved oxygen content in upper intermediate waters in the northeastern Pacific during the late Quaternary are amplified in Santa Barbara basin. Oxygen-poor intermediate water presently flowing into Santa Barbara basin over the western sill has³³ a radiocarbon age of 1,200–1,300 yr compared with 825 yr for the surface mixed layer, which is a mix of California Current and upwelled Pacific Intermediate Water¹². Water as old as 1,980 yr has been reported at depths of $\sim 3,000$ m in the North Pacific³⁴.

If the sediment cycles in Santa Barbara and Guaymas basins were closely linked with changes in ocean circulation during the past 20 kyr (Fig. 1), relations should exist between climate change and age of basin bottom waters. Changes in relative ages of bottom waters are inferred from intrasample differences in radiocarbon ages (uncorrected) between planktonic and benthic foraminifers (Fig. 1). The radiocarbon age differences vary between 40 and 740 radiocarbon years, with the smallest average ^{14}C age differences (~ 100 yr) associated with the Last Glacial Maximum (19.6 kyr) and the Younger Dryas (13–11.2 kyr). In contrast, the average age difference for sample pairs from the Bølling/Allerød interstadial (16.5–13 kyr) is 493 yr. Likewise, during Holocene warmth, radiocarbon age differences, although variable, are relatively high (average 453 yr). Changes in sediment facies are generally correlated to the radiocarbon age differences of basin bottom waters during the past 20 kyr, especially at the beginning and end of the Younger Dryas. In contrast, an increase in radiocarbon age difference (to ~ 550 yr) of bottom waters at the end of the last glacial episode preceded

by ~ 3 kyr the preservation of laminations during the Bølling/Allerød interstadial (Fig. 1).

We believe that the close relations between changes in climate and age of basin bottom waters indicate that the sediment cycles were largely caused by changes in source and age of intermediate waters in the North Pacific. Also, the changes in oxygenation in Santa Barbara basin appear to be nearly synchronous with major changes in North Atlantic Deep Water (NADW) circulation^{1,6}. The Santa Barbara basin was relatively well oxygenated during the Last Glacial Maximum and the Younger Dryas when NADW production was reduced^{1,6} and/or when its southward flow through the Atlantic was at shallower depths^{35,36}. Conversely, when the flux of NADW in the Atlantic was high during the Holocene and Bølling/Allerød warm intervals^{1,6} and/or when it flowed at greater depths^{35,36}, the basin was poorly oxygenated. The stratigraphic records indicate that the switches between the modes were closely synchronized between the North Pacific and North Atlantic.

We suggest two possible mechanism(s) to explain the near-synchronism of changes between the two oceans. The most likely hypothesis is that the inter-ocean palaeoceanographic changes were linked directly through global climate change transmitted through the atmosphere. In this case, cooling during the Last Glacial Maximum and the Younger Dryas increased ventilation of North Pacific Intermediate Waters^{37,40}. This provided a greater contribution of proximal young, well-oxygenated waters to the northeastern Pacific coast margin. The second hypothesis indirectly implicates changes in the strength of thermohaline circulation originating as NADW in the North Atlantic—the so-called oceanic conveyor circulation⁴¹. Although the flow of water within the conveyor from the North Atlantic to North Pacific takes $\sim 1,000$ yr, changes in flux would be propagated rapidly to the North Pacific (J. McWilliams, personal communication). In this model, reduced contributions to the North Pacific of relatively old³⁴ waters originally derived from the North Atlantic led to greater proportions of younger, more oxygenated waters entering Santa Barbara basin. Variations in the age of basin waters (Fig. 1) would result from mixing of the two sources. Irrespective of the process(es) involved, our data indicate the existence of a tight coupling (of circumpolar extent in the Northern Hemisphere) between changes in the atmosphere–ocean–cryosphere during the latest Quaternary. $\square$

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Glacial-interglacial changes in nutrient utilization in the equatorial Pacific Ocean

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THE eastern equatorial Pacific Ocean (EEP) today sustains up to 30% of global marine productivity¹, and the region is one of the largest and most variable marine sources of CO₂ to the atmosphere². This variability is largely controlled by the balance between the physical input of nutrients to the surface ocean and their removal by biological assimilation—the relative nutrient utilization—but the spatial and temporal variability of this balance are poorly understood. Here we use the ¹⁵N/¹⁴N ratio in sedimentary marine organic matter to show strong spatial gradients in relative nitrate utilization throughout the modern EEP. We interpret down-core decline in this ratio through the Last Glacial Maximum (12–24 kyr ago) as a decrease in relative nitrate utilization; the increase in nitrate supply to surface waters due to upwelling during this period was greater than the apparent increase in nitrogen removal by organic matter export out of surface waters. This interpretation is consistent with cooler sea surface temperatures³ and a higher CO₂ flux to the atmosphere^{4,5} during the Last Glacial Maximum, indicating that the EEP surface waters have remained enriched in nutrients, and have not acted as a net sink for CO₂, for at least the past 30,000 years.

A promising method for tracking past changes in relative NO₃⁻ utilization is to determine variations in the ratios of the stable isotopes of nitrogen in sedimentary organic matter^{6,7}. The efficiency with which phytoplankton assimilate NO₃⁻ from near-surface waters is reflected in the $\delta^{15}\text{N}$ values (in ‰ relative to atmospheric N₂; see Fig. 1) of particulate organic matter (POM)^{8,9}, some fraction of which is ultimately preserved in deep-sea sediments. This isotope proxy is based on the observation that POM $\delta^{15}\text{N}$ depends on the $\delta^{15}\text{N}$ of the nitrogenous substrate and on isotope fractionation that occurs during nitrogen uptake by phytoplankton¹⁰. Deep-water NO₃⁻ is the principal source of new nitrogen in the surface ocean. It is the dominant nitrogenous species for new production in the EEP¹¹ where equatorial divergence and strong coastal upwelling commonly result in concentrations in excess of 5 μM (refs 12, 13). In much of this region, upwelled NO₃⁻ has¹⁴ an initial $\delta^{15}\text{N}$ of ~5 to 6‰. But in certain low-oxygen regions within the northern and

southern tropical eastern Pacific, the $\delta^{15}\text{N}$ values can exceed 18‰ because denitrification enriches the remaining NO₃⁻ in ¹⁵N (refs 14, 15).

After NO₃⁻ is delivered to the photic zone, there is preferential uptake of ¹⁴NO₃⁻ by phytoplankton, giving rise to a photosynthate enriched in the light isotope, and to a residual dissolved NO₃⁻ pool that is correspondingly and progressively enriched in ¹⁵N. This has led to the observation of an inverse relationship between the near-surface [NO₃⁻] and the $\delta^{15}\text{N}$ of POM in the

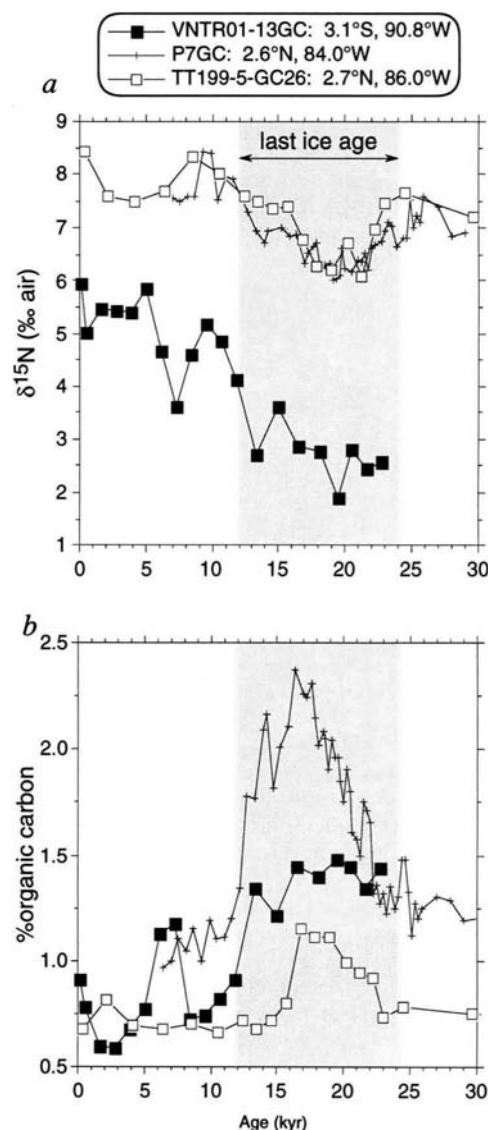
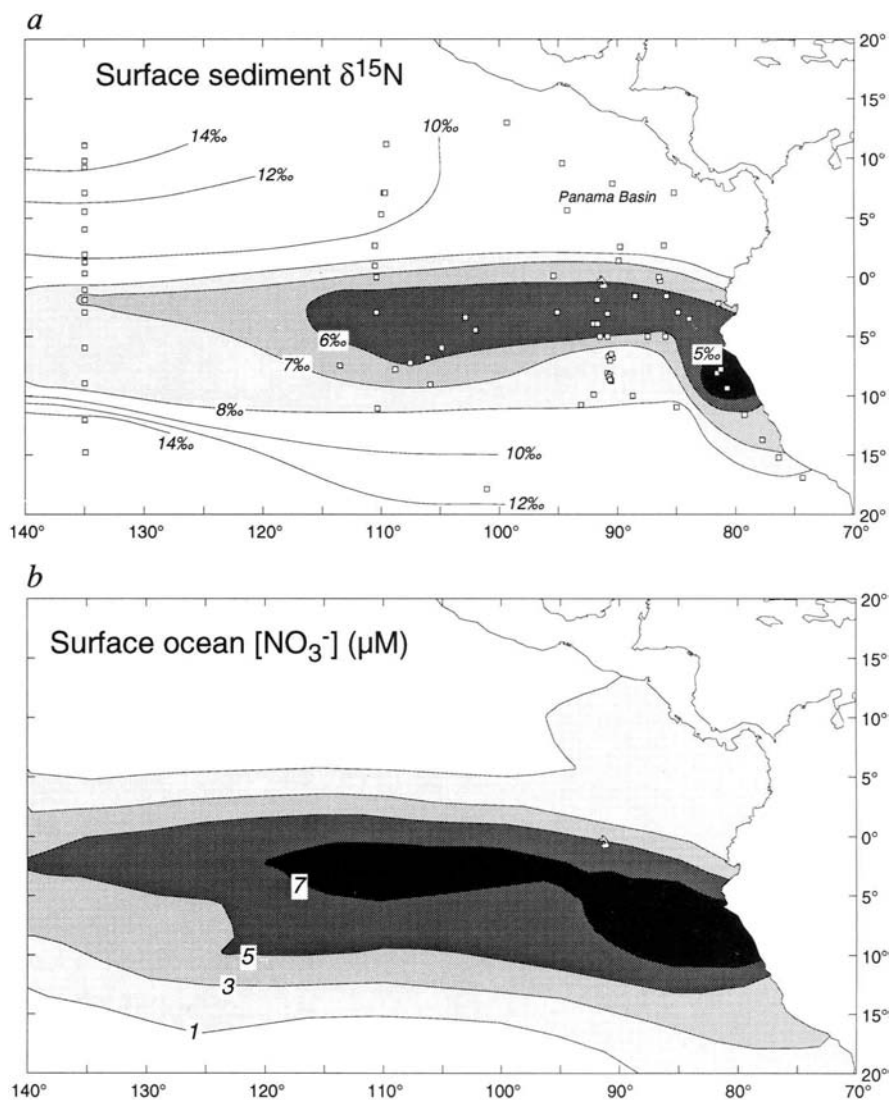


FIG. 1 a, b, Spatial distribution of surface (Holocene) sediment $\delta^{15}\text{N}$ values (a) compared with surface ocean [NO₃]⁻ (b; ref. 13) in the eastern tropical Pacific Ocean. $\delta^{15}\text{N}$ values are from this study (available via e-mail from J.W.F. at jfarrell@brook.edu) and ref. 7. Bulk sediment samples were prepared for $\delta^{15}\text{N}$ analysis by freeze-drying and homogenizing by grinding. Sediments were combusted in an online Fisons NA 1500 element analyser and the evolved N₂ was passed to a VG PRISM isotope-ratio mass spectrometer in a continuous flow of He. Results are reported in the δ notation, $\delta^{15}\text{N} = [(^{15}\text{N}/^{14}\text{N})_{\text{sample}} / (^{15}\text{N}/^{14}\text{N})_{\text{standard}} - 1]$ per mil, relative to atmospheric N₂ and the measurement precision is better than $\pm 0.3\text{‰}$. We have assumed that the isotopic composition of the total nitrogen primarily reflects that of marine organic matter, rather than inorganic nitrogen (ammonium) within clay minerals and adsorbed atmospheric N₂. Initial results from Panama basin sediments indicate that inorganic nitrogen constitutes a minor proportion of the total. As drawn, the $\delta^{15}\text{N}$ isopleths violate 5% of samples by $>0.3\text{‰}$.

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FIG. 2 a, Depth distributions of sedimentary $\delta^{15}\text{N}$ (a) and $\%C_{\text{org}}$ (b) in three cores from the eastern equatorial Pacific Ocean. The age models are based on oxygen-isotope stratigraphies²⁶ and unpublished data. Percent C_{org} values for P7GC were determined via Leco carbon analysis²⁶. The $\%C_{\text{org}}$ values in cores VNTR01-13GC and TT199-5-GC26 were calculated as the difference between total carbon, determined on an elemental analyser (Carlo Erba 1500NA NCS), and inorganic carbon determined with a CO_2 coulometer (Coulometrics 5011). Precision of $\%C_{\text{org}}$ determinations is $\pm 3\%$. The same pattern of high organic-carbon values during the LGM and low values during the Holocene is observed if the $\%C_{\text{org}}$ data are expressed as C_{org} accumulation rates ($\text{g cm}^{-2} \text{ kyr}^{-1}$) because sedimentation rates were also higher during the LGM.



water column¹⁶. Similar associations have been observed between $[\text{NO}_3^-]$ and the $\delta^{15}\text{N}$ of organic matter collected in sediment traps and preserved in the underlying sea-floor sediments⁷. These associations have been seen in several oceans on both temporal and spatial scales^{6,7,9,16,19}. As the $\delta^{15}\text{N}$ of subsurface NO_3^- is generally considered to be uniform in the deep ocean, outside denitrification regions⁷, the associations between $[\text{NO}_3^-]$ and POM $\delta^{15}\text{N}$ have been ascribed mainly to variations in relative NO_3^- use.

A comparison of surface sediment $\delta^{15}\text{N}$ and surface ocean $[\text{NO}_3^-]$ reveals that a $\delta^{15}\text{N}$ range of $\sim 11\text{‰}$ (from 16.4 to 4.8‰)—which exceeds our analytical uncertainty by 35 times—corresponds to a $[\text{NO}_3^-]$ range from zero to $>7 \mu\text{M}$ (Fig. 1). The spatial distributions of $\delta^{15}\text{N}$ and $[\text{NO}_3^-]$ values are strikingly similar and inversely related. Three distinct distribution patterns are observed in the $\delta^{15}\text{N}$ map. The most conspicuous pattern is meridional and is characterized by highest $[\text{NO}_3^-]$ values and lowest $\delta^{15}\text{N}$ values centred within the 0° to 5° S latitudinal band, and by progressively lower $[\text{NO}_3^-]$ values and higher $\delta^{15}\text{N}$ values to the north and south. This pattern is especially distinct in the offshore region where upwelling associated with the equatorial divergence system is a direct influence. The second distribution pattern is an east-to-west, or zonal, gradient where the central equatorial Pacific Ocean shows a higher $\delta^{15}\text{N}$ and is lower in $[\text{NO}_3^-]$ than in the EEP. The zonal gradient, although weaker than the meridional one, extends across a larger distance and may span the entire equatorial Pacific according to sediment

data from the Ontong Java plateau (M. Altabet, personal communication). The third distribution pattern is centred in the highly productive waters off Ecuador and Peru, and is associated with the strong coastal upwelling regime. This region is marked by the lowest $\delta^{15}\text{N}$ values measured in this study and by the highest $[\text{NO}_3^-]$ values. Isotopic values increase outwards from this region where a decrease in upwelling intensity causes the nutrient content of the surface waters to decline, and water temperatures to rise.

All three distribution patterns have a common origin. Phytoplankton growth is limited by factors other than NO_3^- availability at upwelling centres, where $[\text{NO}_3^-]$ is highest. When there is incomplete use of an abundant supply of NO_3^- , ^{14}N -enriched POM is formed in accordance with first-order Rayleigh fractionation kinetics. As upwelled waters are advected north and south from the equatorial belt, and west from the coastal upwelling cells, phytoplankton continue to use the remaining NO_3^- . This progressive drawdown of NO_3^- is accompanied by ^{15}N -enrichment of the residual NO_3^- pool and of the POM, with the latter ultimately sinking to the sea floor as physical aggregates, faecal pellets or marine snow. It is during this transformation of POM to sea-floor sediment that organic-matter degradation, trophic transfer and early diagenesis occur^{19,20}. A rather constant 3‰ increase in $\delta^{15}\text{N}$ from sinking POM to surface sediments in the central equatorial Pacific Ocean was noted in a previous study²¹. The nature of this diagenetic offset is poorly understood, but it clearly does not obliterate the isotopic signal generated in the

surface ocean. Coherent phasing in down-core records of $\delta^{15}\text{N}$ and palaeoceanographic variables such as $\delta^{18}\text{O}$, further suggest that climate-related variations in $\delta^{15}\text{N}$ are faithfully preserved, although offset from surface POM values^{6,7,17,22}.

In the Panama basin region, the moderately high and relatively uniform $\delta^{15}\text{N}$ values of 8–10‰ are attributed to a combination of denitrification in the subsurface waters¹⁵ and to high NO_3^- utilization which results in relatively low $[\text{NO}_3^-]$ (<2 μM) in the surface waters. There is a shallow and particularly intense oxygen minimum in this region which fosters ^{15}N -enrichment of the subsurface nitrate¹⁵. The $\delta^{15}\text{N}$ of NO_3^- entering the photic zone from below may range¹⁵ between 10 and 20‰, but vertical profiles of nitrate $\delta^{15}\text{N}$ indicate photic zone values of 5–11‰, reflecting the mixture of isotopically heavy NO_3^- from below and lighter NO_3^- advected by surface currents. Assimilation of the ^{15}N -enriched NO_3^- results in ^{15}N -enriched POM and similarly elevated sediment $\delta^{15}\text{N}$ values.

As denitrification also occurs in the coastal regions off South America²³, ^{15}N -enriched sediment might be expected to occur there. In fact, the $\delta^{15}\text{N}$ of NO_3^- in the near-surface waters off Peru is slightly heavy, ranging²³ between 5 and 10‰. But unlike in the Panama basin, waters off South America have high $[\text{NO}_3^-]$ and the resultant relative NO_3^- use, together with a shorter food chain (fewer trophic levels^{20,24}), probably accounts for the light $\delta^{15}\text{N}$ values of the Peru margin sediments.

The similarity of the patterns in the maps of $\delta^{15}\text{N}$ and $[\text{NO}_3^-]$ confirms previous results^{7,19}, and extends this inverse relationship over a significantly broader area of the Pacific. This relationship also bolsters both the interpretation that sediment $\delta^{15}\text{N}$ is inversely linked to near-surface $[\text{NO}_3^-]$ and the hypothesis that the dominant control of sediment $\delta^{15}\text{N}$ is progressive NO_3^- utilization with distance from the locus of upwelling⁷. The quantitative link of $[\text{NO}_3^-]$ to sedimentary $\delta^{15}\text{N}$ is not obscured by such complicating factors as denitrification, organic-matter degradation and early diagenesis.

If the relationship observed between Holocene sediment $\delta^{15}\text{N}$ and relative NO_3^- utilization extends back in time, then past changes in the nutrient supply-demand balance should be provided by down-core records of sediment $\delta^{15}\text{N}$. Two profiles from the Panama basin and one from the Galápagos region show a consistent 1 to 2‰ decrease in $\delta^{15}\text{N}$ from the Holocene (0–12 kyr ago) to the Last Glacial Maximum (LGM) (Fig. 2a). The $\delta^{15}\text{N}$ values return towards Holocene levels during the transition from the LGM to the preceding interglacial. There is a consistent ~3 to 4‰ $\delta^{15}\text{N}$ offset between the Galápagos profile and the Panama basin records because of higher relative NO_3^- utilization in that basin and the upwelling of ^{15}N -enriched NO_3^- due to denitrification.

We attribute the decline in $\delta^{15}\text{N}$ during the LGM at all three sites to a decrease in relative NO_3^- utilization. In other words, the physical supply of NO_3^- exceeded the biological demand even more than it does today. This interpretation is supported by evidence for increased upwelling during the LGM^{3,25}, which would have augmented the NO_3^- surplus and exacerbated the high-nitrate, low-chlorophyll conditions observed today². However, biological demand for NO_3^- is also thought to have increased during the LGM. Indeed, a several-fold increase in both the sedimentary concentration and accumulation rate of organic carbon in the EEP (Fig. 2b) have been attributed to an increase in export production from the surface waters^{4,26,27}. This increase in productivity, initiated perhaps by iron fertilization²⁸, would have resulted in a greater demand for NO_3^- . Nevertheless, the lighter $\delta^{15}\text{N}$ values during the LGM strongly suggest that the supply continued to dominate. Using the relationship between the isotopic compositions of sedimentary N and source NO_3^- , the fractionation factor (assumed to be 5‰) and the extent of NO_3^- utilization²⁹, it can be shown by calculation that relative NO_3^- utilization fell by about 10 and 30% and $[\text{NO}_3^-]$ was ~25 and ~60% higher in the Panama basin and in the Galápagos region, respectively, in the LGM compared with the

Holocene. These results are further supported by estimates of lower sea surface temperatures³ and greater CO_2 efflux during the LGM^{4,5}.

In arriving at our preferred interpretation for the LGM decline in $\delta^{15}\text{N}$, we considered four other factors that could have affected the sedimentary $\delta^{15}\text{N}$ record. First, a glacial-interglacial change in plankton ecology, such as an increase in the relative abundance of diatoms over coccoliths, could have resulted in lower $\delta^{15}\text{N}$ during the LGM because diatoms may fractionate NO_3^- more than coccoliths³⁰. But this ecological scheme is considered of minor importance because the sedimentary record does not show a clear glacial-interglacial pattern of opal accumulation in the EEP²⁷. Second, the $\delta^{15}\text{N}$ value of NO_3^- that wells up into the photic zone may have varied on a glacial-interglacial timescale³¹. Indeed, recent evidence suggests that water-column denitrification in some regions was substantially reduced during the LGM^{29,32}. If this decline were significantly large, it might have decreased the $\delta^{15}\text{N}$ of NO_3^- in the mean global deep water from the modern value of 5 or 6‰, thus explaining the lighter values observed in the Pacific sedimentary profiles. The fact that $\delta^{15}\text{N}$ values from some oceanic regions increased during the LGM argues against this possibility⁶. Third, sedimentary $\delta^{15}\text{N}$ may have been lowered by a change in the diagenetic offset during the LGM. The correspondence between lower $\delta^{15}\text{N}$ and higher organic-carbon concentrations (% C_{org}) in the down-core records would then be taken as evidence of a reduction in organic degradation and thus less preferential removal of ^{14}N (ref. 33). This is unlikely, however, because the surface sediment data show no significant relationship between $\delta^{15}\text{N}$ and % C_{org} . Furthermore, in other Pacific regions, the opposite is observed; higher $\delta^{15}\text{N}$ is correlated with higher % C_{org} in cores from the northwest Mexican margin³². Last, a significant increase in the input of terrestrial organic matter during the LGM, which would have contributed to lighter $\delta^{15}\text{N}$ values, is discounted because profiles of the sedimentary carbon:nitrogen ratio change very little over the Holocene-LGM transition⁴.

The balance of evidence strongly suggests that the $\delta^{15}\text{N}$ values of marine organic matter in open-ocean sediments reflect the $[\text{NO}_3^-]$ in the overlying surface waters and provide a measure of relative NO_3^- utilization. Larger 'excess' NO_3^- concentrations, thus lower relative use, in the EEP during the LGM are consistent with cooler sea surface temperatures, greater upwelling, and an even higher CO_2 efflux. Despite higher export productivity in the area during the LGM^{4,27}, the surface waters remained nutrient-rich. The EEP was therefore not a site of net CO_2 uptake during the glacial period. □

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Tradeoff between resistance and tolerance to herbivore damage in a morning glory

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MANY plant characters, including toxic secondary compounds, trichomes, spines and tough, nutrient-poor leaves have evolved at least in part as defences against pathogens and herbivores, including phytophagous insects^{1–6}. Models of the evolution of resistance^{7,9} predict that allocation to defence is determined by a tradeoff between the benefits of resistance, such as reduction in herbivore damage, and costs of resistance, generally envisaged as reduction in fitness in an environment in which herbivores are absent⁹. However, despite attempts to determine the costs of resistance, there is little convincing evidence that they exist and constrain the evolution of defences^{10,11}. Here we report the existence of such a cost in the tall morning glory, *Ipomoea purpurea*: genotypes that exhibit relatively high levels of resistance to insects that cause damage to apical meristems exhibit relatively low tolerance to this form of damage. We also show how this type of tradeoff constrains the evolution of resistance.

In a field experiment, ten inbred lines exhibited heterogeneity in damage caused by generalist herbivores, flea hoppers and insects causing damage to the apical meristem (Fig. 1). There

thus appears to be considerable genetic variation for resistance to each of these herbivores in the population from which the lines were established. Moreover, line-mean correlations between resistance to apical meristem damage and resistance to each of the other herbivores are low and non-significant, indicating that resistance to apical meristem damage involves different mechanisms, and thus is a different character, from resistance to the other herbivores (correlation coefficients $r=0.22$ and $r=-0.27$ for correlations with resistance to generalist insects and to flea-hoppers, respectively). These results are consistent with previous investigations of *Ipomoea purpurea*^{9,12}, in which genetic variation was detected for resistance to generalist herbivores and other types of insects.

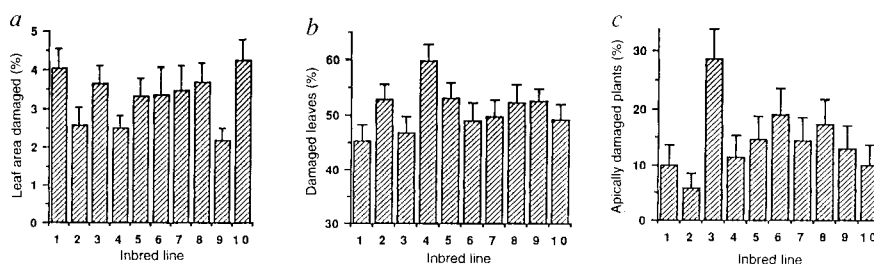
In a second experiment, we measured the tolerance to apical damage of 12 inbred lines. Tolerance differs from resistance in that it is the amount by which fitness is reduced for a given amount of damage, whereas resistance is the amount of damage a plant experiences for a given abundance of herbivores¹³. We measured tolerance to simulated herbivore feeding in a greenhouse, thus eliminating natural herbivore completely. Moreover, we investigated damage to the apical meristem because it is easy to mimic experimentally.

A multivariate analysis of variance (MANOVA) showed that lines varied overall in the five fitness components examined (main effect of line, $F=7.7$, d.f. = 55, 78, $P<0.0001$), and that damage treatment caused a significant reduction in these fitness components (main effect of damage treatment $F=10.2$, d.f. = 10, 32, $P<0.0001$). Most importantly, however, a significant treatment by line interaction ($F=1.5$, d.f. = 100, 375, $P<0.0025$) showed that inbred lines varied in their overall fitness response to the damage treatment, indicating that lines varied in their degree of tolerance to apical damage.

Univariate analyses (Table 1) of individual fitness components revealed that the MANOVA results originated largely from vari-

FIG. 1 Variation among inbred lines in percent-age damage by damage type. a, Generalist insect herbivores: several grasshopper and lepidopteran species ($F_{9,538}=1.99$, $P<0.05$). b, Flea hoppers ($F_{9,538}=1.96$, $P<0.01$). c, Insects damaging apical meristem ($G=19.2$, d.f. = 9, $P<0.05$). For a and b, probabilities indicate significance of line effect in analysis of variance. For c, probability reflects significance of inbred line effect in a G-test. The effect of female parent nested within line was non-significant for generalist insects and for flea hoppers ($F_{10,38}=0.56$ and 0.99, respectively), indicating minimal maternal effects on resistance. The effect of female parent could not be tested for damage to apical meristem.

METHODS. Ten inbred lines were selected randomly from a set of 35 lines inbred by selfing for 7 generations, each generation being propagated by a single selfed seed. The progenitors of all lines were collected from the same population in Durham County, NC. To produce the experimental seed, two selfed offspring seed from a single seventh-generation plant were chosen as 'female parents' from each line. Each of these female parents was allowed to produce 35 experimental seeds by selfing. These experimental seeds were germinated in the greenhouse and then transplanted to a tilled field when they had produced



cotyledons. Seedlings were planted in the field in a randomized block design. Four weeks later, plants were censused for damage by several types of herbivores: damage caused by generalist herbivores was estimated as the number of 0.694 cm² transparent grid squares (to the nearest quarter square) covering the area of damage on each leaf and expressed as the proportion of total leaf area for each plant. Flea hopper damage was estimated as the presence or absence of damage on each leaf and expressed as the proportion of leaves affected. Plants were also scored for the presence or absence of damage to the apical meristem. (See ref. 25 for further details.)

TABLE 1 Univariate analysis of variance for plant performance characters

Source	df	Growth rate		First flower		First Capsule		Flower number		Capsule number	
		SS	F	SS	F	SS	F	SS	F	SS	F
Block	4	863.4	16.7***	138.3	5.4***	88.3	2.86*	74.0	1.47	1,827.6	10.2***
Line	11	924.0	6.5***	1,268.5	18.2***	1,300.3	15.3***	1,934.9	8.41***	16,425.0	18.6***
Damage	2	218.4	8.5***	371.2	29.2***	300.6	19.5***	34.5	0.83	491.6	3.1†
Block × Line	44	368.1	0.7	256.5	0.9	398.8	1.2	427.5	0.77	2,335.6	1.2
Block × Damage	8	103.0	1.0	34.6	0.7	51.8	0.8	60.9	0.61	109.9	0.3
Line × Damage	20	318.5	1.2	149.8	1.2	148.6	1.0	418.2	1.67†	1,609.2	1.8*
Error	80	1,032.3		508.0		616.7		1,004.0		3,588.1	

The other terms in the MANOVA for the 5 performance variables were: block, d.f. = 20, 253, F -ratio = 5.5, $P < 0.001$; block × line, d.f. = 220, 383, $F = 1.1$, P non-significant; and block × treatment, d.f. = 40, 334, F -ratio = 0.8, P non-significant. For all three terms, the model error was used as the error term. For the univariate analyses reported here, F -ratios were calculated using the error mean square in all cases except flower and capsule number, in which the line × damage sum of squares (ss) was used as a denominator for the main effects of line and damage. Type III sums of squares were used. The tolerance experiment used 12 inbred lines, including 6 that were also used in the field experiment. Seedlings were allocated to three treatments: (1) early damage at the seedling stage; (2) late damage at the rapid growth stage; (3) control. Three seeds from each of five females for each inbred line were grown individually in 4-inch pots and placed on greenhouse benches in a randomized block design, with three plants of each line per block. One of the plants from each line in each block was assigned to each of the treatments. Early-damage plants had the extreme tip removed at the seedling state (17 days after planting), late-damage plants had the growing tip removed during the rapid growth phase (4 weeks after planting), and control plants were not damaged. Plants were allowed to grow, flower and set seed, and the following fitness components were assessed: growth rate, date of first flowering, date of first capsule maturation, total flower production and total capsule production. Growth rate was estimated as the difference in estimated leaf area measured at 4 weeks and at 17 days, divided by that measured at 17 days. Total flower production was estimated from 11 counts performed over a six-week period. Individual flowers last for one day. Tolerance was estimated for each inbred line as the ratio of the mean number of capsules produced by plants damaged at four weeks to the mean number produced by the undamaged control plants. This ratio was based on the late-damage treatment because it best corresponded to the timing of damage measurement in the field experiment. (See ref. 25 for further details.) † $P < 0.1$, * $P < 0.05$, *** $P < 0.001$.

ation among lines in the effect of apical damage on flower and seed capsule production. Because seed production is highly correlated with capsule production ($r = 0.98$) (ref. 14), these results indicate that lines vary in tolerance, at least as measured in terms of the female component of total fitness. There was no detectable difference among inbred lines in the effect of damage on plant growth rate, or on the timing of initial flowering or capsule production.

Because six inbred lines were common to both the field and greenhouse experiments, we could determine whether resistance to apical damage and tolerance of apical damage are related. Line means for these two traits are strongly negatively correlated (Fig. 2; $r_s = -0.943$, $P < 0.02$), indicating a strong negative genetic correlation between these two traits. Such a correlation is the embodiment of a tradeoff^{15,17}, as it indicates that selection for increased resistance should lead to decreased tolerance. By using an analysis based on inbred lines we were not able to separate the additive and non-additive components of covariance between resistance and tolerance, but previous investigations of several types of resistance in *I. purpurea*^{9,12} have indicated that most variation for resistance is additive, suggesting that the covariance observed involves primarily additive variance for resistance to meristem damage. Because additive and non-additive variance are uncorrelated¹⁸, this suggests that the measured covariance is primarily due to additive genetic effects.

Several theoretical treatments have examined the effects of tradeoffs between the benefits (reduced herbivore damage) and costs (reduced fitness in the absence of herbivores) of resistance on the evolution of resistance⁷⁻⁹. In general these models, which assume that fitness costs of resistance are either 'self-toxicity', 'allocation' or 'ecological' costs¹¹, yield three important predictions for plausible cost and benefit functions: (1) there is a single peak in the adaptive landscape, so either net selection on resistance is directional (meaning that either no resistance or complete resistance is favoured) or it is stabilizing (meaning that an intermediate level of resistance is favoured); (2) an increase in herbivore abundance causes an evolutionary increase in the mean level of resistance; and (3) reduction in the efficacy of defence by herbivore counteradaptation causes an evolutionary change in the level of resistance. Tradeoffs between resistance and tolerance, however, may have different implications for the evolution of resistance, as can be seen from the following model.

Previous models of the evolution of resistance infer the evolutionary equilibrium for allocation to resistance from the position of the peak(s) in an adaptive landscape, in which plant fitness, W , is represented by the relationship

$$W(R) = W_0 - C(R) - D(R)T, \quad (1)$$

where W_0 is the fitness of a plant with no resistance in the absence of herbivores, R is the proportion of the maximum possible allocation of resources to resistance, $C(R)$ is the cost of resistance, $D(R)$ is amount of damage experienced by a plant with allocation R , and T is the reduction in fitness per unit damage. T is essentially a measure of tolerance, which is assumed to be the same for all values of R .

To incorporate the observed inverse relationship between resistance and tolerance in *I. purpurea*, this equation can be modified by allowing T to be a function of R . If, as suggested by Fig. 2, tolerance is inversely related to resistance, this may be represented by

$$T(R) = K_1 + cR. \quad (2)$$

Large values of $T(R)$ indicate low tolerance (damage causes a

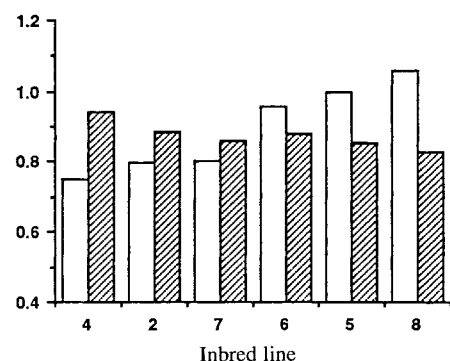


FIG. 2 Line means for resistance to and tolerance of apical meristem damage for the six inbred lines common to field and greenhouse. The y axis is proportion damage (for resistance bars shaded) or proportional reduction in fitness (for tolerance bars white). Line numbers correspond to lines portrayed in Fig. 1. Spearman's rank correlation, $r_s = -0.943$, $P < 0.02$.

large reduction in fitness), and small values indicate high tolerance (damage causes little reduction in fitness).

As in the previous models, as allocation to resistance increases, the amount of damage experienced by a plant must asymptote towards zero⁹, which can be represented by

$$D(R) = D_0 e^{-bR}, \quad (3)$$

where D_0 is the amount of damage to plants with no resistance ($R=0$) and b is a constant representing the efficacy of resistance at reducing damage. Combining equations (1)–(3) yields

$$W(R) = W_0 - C(R) - (K_1 + cR)D_0 e^{-bR}. \quad (4)$$

Analysis of this model indicates that a cost of resistance in the form of a tradeoff between resistance and tolerance may constrain the evolution of resistance differently from standard allocation or ecological costs in at least three ways.

(1) There may be two peaks in the adaptive landscape, corresponding to maximal resistance and no tolerance, and to no resistance and maximal tolerance (Fig. 3a). For a given abundance of herbivores, increased resistance is favoured only if mean allocation to resistance is initially greater than a threshold corresponding to the minimum in Fig. 3a; otherwise, decreased resistance, accompanied by increased tolerance, will be favoured. Different levels of resistance and tolerance may thus evolve in different populations, even though the environmental conditions are similar.

(2) When two such peaks exist, an increase in herbivore abundance is generally not expected to cause an evolutionary increase in resistance. This expectation arises because, unless the increase in herbivore abundance is very large, there will be no change in the position of the peaks. The adaptive landscape remains concave upwards, and only the position of the fitness minimum may change (Fig. 3a). In the previous models, although the cost of resistance is fixed, when herbivore abundance increases the benefit of resistance also increases because more herbivores are deterred. The benefit thus increases relative to the cost, and it is profitable to increase allocation to resistance. By contrast, in equation (4) the benefits of tolerance also increase with herbivore abundance. The 'cost' of resistance, in this case absence of tolerance, also increases, tending to maintain both maximal tolerance and maximal resistance as alternative evolutionary equilibria.

(3) Counteradaptation to plant defenses by a herbivore, which in effect decreases the efficacy of resistance, b , tends not to influence allocation to resistance (Fig. 3b). Changing b often alters only the fitness minimum, but does not affect the alternative equilibria, as it does in the previous models.

These considerations indicate that the type of tradeoff we have identified is likely to constrain the evolution of resistance in ways very different from those previously envisaged. Although we have considered in detail here a model in which the relationship between resistance and tolerance is linear, similar results are obtainable from models in which this relationship is not linear (for example, quadratic). In particular, analysis of such models indicates that results (1)–(3) above can easily occur (see Fig. 3a legend for criteria for multiple peaks; a more detailed analysis will be presented elsewhere). We emphasize, however, that in both the linear and nonlinear cases, some parameter combinations yield results similar to those of the previous models (for example, when the slope of the regression of tolerance on resistance, c_1 , is low, or when ecological/allocation costs of resistance, K_2 , are high). The general evolutionary implication of a tradeoff between resistance and tolerance is that modifying the previous models by simply incorporating a tradeoff of the type reported here yields a more diverse array of possible evolutionary outcomes.

The observation that most inbred lines tested exhibited intermediate levels of resistance to apical damage (Fig. 1) may appear to contradict the prediction of our model that resistance should evolve to be minimal or maximal. However, the values of the

model's parameters are currently unknown for *I. purpurea*, and they may lie in the region of parameter space that corresponds to stabilizing selection. Alternatively, there may be additional constraints acting on the evolution of resistance in *I. purpurea* that are not accounted for in our model.

Although other constraints may be operating, it is clear that the evolution of resistance to insects attacking the apical meristems of *I. purpurea* cannot be completely understood without considering the simultaneous evolution of tolerance to these insects. Although tolerance is often assessed in forage and agricultural plants^{19–23}, evolutionary biologists have seldom consid-

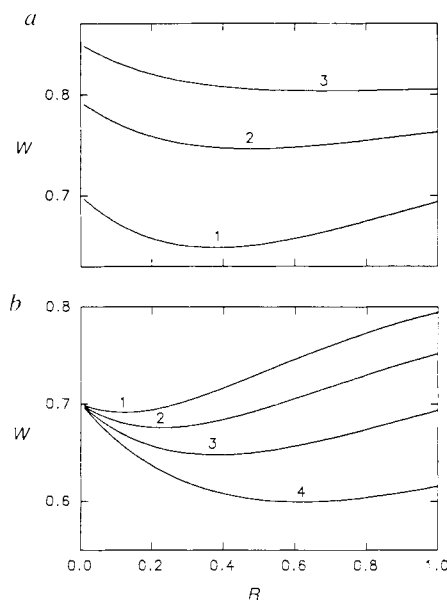


FIG. 3 Examples of two-peaked fitness landscapes for resistance. Lines are plots of relative fitness, $W(R)$, versus proportional allocation to resistance, R , using equation (4). Fitness values are all made relative to a fitness of 1 for genotypes with no resistance ($R=0$) in the absence of herbivores (that is, to $W_0=1$). a, Changing herbivore abundance, as reflected in the parameter D_0 , alters the position of the fitness minimum but does not alter the position of the fitness peaks (equilibria). In this example, for all plots, $K_2=0.083$, $c=0.7$, and $b=1.5$. Lines labelled 1, 2 and 3 correspond to D_0 values of 1.0, 0.69 and 0.5, respectively. Labels are positioned along the x axis at the minimum of the curve. In general, the variables in equation (4) may be rescaled so that $T(R=1)=1$. Then $K_1=1-c$. Let the cost, C , of resistance be linearly related to resistance, so that $C(R)=K_2R$. Then equation (4) becomes $W(R)=W_0-K_2R-((1-c)+cR)D_0e^{-bR}$. The conditions for two peaks are $(dW/dR)_{R=0}<0$ and $(dW/dR)_{R=1}>0$, which is equivalent to the condition $(b-\alpha)/(1+b)<c-b\alpha e^b$, where $\alpha=K_2/D_0$. This condition can be satisfied as long as $(b-\alpha)/(1+b)<b-\alpha e^b$, which is possible as long as α is sufficiently small. For example, for b values of 0.5, 1.0 and 2.0, this condition will be satisfied as long as α is less than 0.17, 0.225 and 0.188, respectively. Thus, in general, the existence two peaks is possible as long as the cost of resistance is small compared to the amount of damage incurred by plants with no resistance. By contrast, when there is no cost of resistance (that is, $K_2=0$), $d^2W/dR^2=bD_0e^{-bR}(1+c+cR)$, which is always ≥ 0 . Consequently, stabilizing selection is not possible without a cost of resistance, and will be unlikely when the cost of resistance is small. When $K_2=0$, differentiating equation (4) with respect to R and solving for the equilibrium, R , gives $\bar{R}=(1/b)-(1-c)/c$. Because this relationship does not involve D_0 , changes in herbivore abundance, which affect D_0 , will not change the position of the fitness minimum. When K_2 is small, that is, when physiological costs of resistance are small, this result should hold approximately, as shown in a. A nonlinear relationship between tolerance and resistance can be modelled by allowing equation (2) to be $T(R)=K_1+c_1R+c_2R^2$. Then the conditions for two fitness peaks are $[(1-c_1-c_2)b]<\alpha<e^{-b}(b-c_1-2c_2)$. b, Changing efficacy of resistance, b , alters the position of the fitness minimum, but does not alter the position of the fitness peaks. In this example, for all plots, $\alpha=0.083$ and $c=0.7$. Lines 1, 2, 3 and 4 correspond to $b=2.1, 1.8, 1.5$ and 1.2 , respectively.

ered it explicitly as a trait that may be involved in plant-herbivore coevolution. Moreover, a tradeoff between resistance and tolerance is not likely to be detected by standard assays used for assessing costs of resistance, which involve growing genotypes differing in resistance in a common environment lacking herbivores and determining whether there is a negative genetic correlation between resistance level and fitness^{9,11,17,24}. However, the cost of resistance engendered by the negative correlation with tolerance arises because plants that are slightly resistant experienced damage, and the negative effect of that damage on fitness is large. Because the cost in this case (reduced tolerance) is expressed only in the presence of herbivory, eliminating herbivores in the standard assay would preclude the detection of that cost. Therefore, previous experiments that have failed to detect allocation costs associated with resistance^{9,11,12,14} cannot rule out the existence of tradeoffs between resistance and tolerance. Thus we do not yet know how common such tradeoffs are in natural populations. □

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Mutual policing and repression of competition in the evolution of cooperative groups

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EVOLUTIONARY theory has not explained how competition among lower level units is suppressed in the formation of higher-level evolutionary units^{1,2}. For example, the key problem of early evolution is how small, individual replicators formed cooperative groups of sufficient complexity to allow accurate copying of the genetic material³. The puzzle is why parasites did not subvert the formation of cells by obtaining benefits from the group without contributing to shared traits that enhance reproduction⁴. These parasites would outcompete other replicators within the cell, disrupting reproductive fairness among subunits and destroying the functional coherence of the group. A similar problem arose at a later evolutionary stage with the orderly mendelian segregation of subunits (chromosomes) within cells, and reproductive fairness continued to be a problem in the evolution of insect⁵ and human societies⁶. Here I present a simple model to show how reproductive fairness evolves among subunits to create functional coherence and higher-level units. Self-restraint, which evolves according to the kin-selection coefficient of relatedness, is not sufficient: mutual policing and enforcement of reproductive fairness are also required for the evolution of increasing social complexity.

Competition within groups can have both benefits and costs for an individual. If resources are limited within the group, the most competitive individuals will gain a disproportionate share of the local benefits. However, competition often reduces the group's overall efficiency in using local resources, thus lowering the average success of the group members. A simple model describing these costs and benefits of group competition⁷ is:

$$w_{ij} = (z_{ij}/z_i) (1 - z_i) \quad (1)$$

where w_{ij} and z_{ij} are the fitness and competitive intensity, respectively, for the j th individual in the i th group, and z_i is the average

competitive intensity for members of the i th group. Each individual gains a share z_{ij}/z_i of the local resources, but higher levels of competition reduce the average group productivity, $1 - z_i$.

The model captures the essential tension between individual and group success. For example, the individuals may be parasites and the local resource may be food obtained from the host. Parasites compete within a host by increasing the rate at which host tissues are exploited and consequently damaged. If z_{ij} is the rate of exploitation by an individual parasite, then greater exploitation leads to greater relative success within the host, z_{ij}/z_i . However, rapid exploitation may damage the host, thus reducing the total food available to the parasites by an amount $1 - z_i$.

The equilibrium for the model in equation (1) can be found by maximizing w_{ij} with respect to variants in z_{ij} (see the legend of Fig. 1 for details). The equilibrium is $z^* = 1 - r$, where r is the kin-selection coefficient of relatedness among group members. Self-restraint evolves when relatedness is high, reducing competition among group members and increasing group success. By contrast, low relatedness leads to intense competition and low group productivity. In the parasite example, decreasing relatedness causes greater damage (virulence) to the host⁸.

Self-restraint favoured by kin selection promotes improved efficiency of resource utilization. However, there remains a gap between selectively favoured behaviour and complete functional coherence. The gap occurs because within-group competition is increasingly favoured as r declines. In many cases, the genetic relatedness among subunits is low because of mixing among groups and mutation. In addition, many of the important transitions required cooperation between different kinds of units. For example, cooperative symbiosis among different 'quasispecies' of replicators is believed to be essential for the early evolution of genetic systems and the first protocells³. The orderly patterns of mendelian segregation are spectacularly rigid controls on the relative success of the different kinds of subunits (chromosomes) within the cell. In many social insects the relatedness among workers is low, and the workers can subvert the cooperative coherence of the colony by laying their own eggs rather than raising those produced by the queen. Thus kin selection alone is unlikely to explain transitions to new evolutionary units or more complex cooperative groups.

If competition within the group can be repressed then the success of each group member would be increased. Reduced

competition would be particularly valuable when relatedness and self-restraint are low. But how can traits that reduce competition evolve when individuals gain by struggling for a larger share of the local resources? This problem is similar to the famous 'tragedy of the commons', apparently first described in formal economic terms in 1833 by William Forster Lloyd⁹ (see refs 10, 11). The difficulty is that each individual gains by pursuing interests that increase returns relative to neighbours and decrease the value of common goods. Group-level efficiency is unlikely unless some mechanism exists to repress competition and promote fair distribution of resources.

Kin selection and repression of internal competition can each favour group coherence and the evolution of higher-level units. My intention is to clarify the interaction between these two fundamental processes.

Consider an extension of the previous kin-selection model. A second trait, a , determines each individual's contribution to a mechanism that reduces competition among all members of the local group (mutual policing). The extended model is:

$$w_{ij} = [a_i - ca_{ij} + (1 - a_i)z_{ij}/z_i][1 - (1 - a_i)z_i] \quad (2)$$

where a_{ij} is an individual's contribution to mutual policing, which has a cost to the individual of ca_{ij} . The average level of policing in the local group is a_i . Each potentially competitive interaction is reduced in both opportunity for gain by the victor, $(1 - a_i)z_{ij}/z_i$, and damage to local resources, $(1 - a_i)z_i$.

We can obtain an approximate description of evolutionary trends for competitiveness and policing, z and a , by examining the gradient of w_{ij} with respect to variants in z_{ij} and a_{ij} . As explained in the legend of Fig. 1, this gradient is given by

$$\partial w / \partial z = (1 - a)[(1 - (1 - a)z)(1 - r)/z - r(1 - ca)] \quad (3)$$

$$\partial w / \partial a = rz(1 - ca) - c(1 - (1 - a)z) \quad (4)$$

where, as before, r is the coefficient of relatedness among members of the local group. The system moves towards one of two equilibria. When $r > 1 - c$, the system tends towards $a^* = 0$ and $z^* = 1 - r$, the same equilibrium for the case in which no policing occurs (Fig. 1a). When $r < 1 - c$, the system tends towards complete repression of competition, $a^* = 1$ and $z^* = c/[r(1 - c)]$ (Fig. 1b).

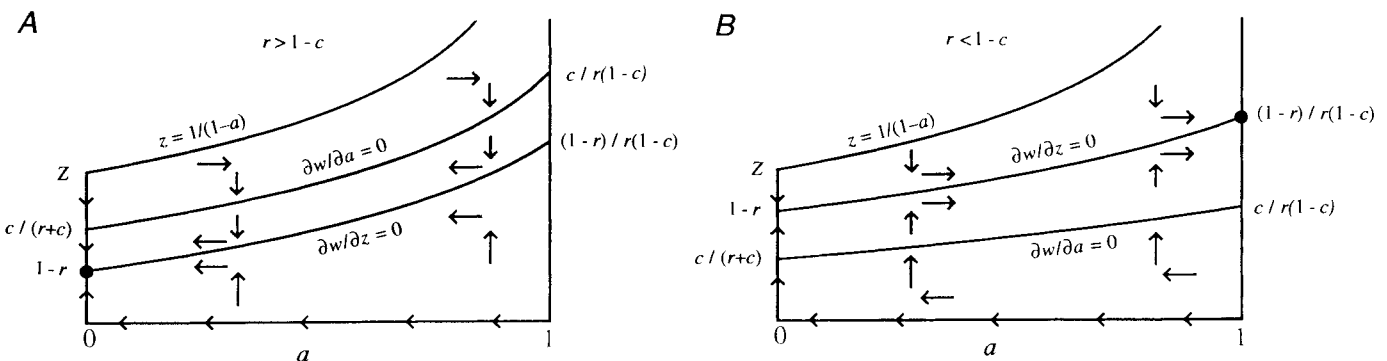


FIG. 1 Evolutionary dynamics of competitiveness, z , and mutual policing, a . In A, when relatedness is high relative to the benefits of mutual policing, $r > 1 - c$, then the outcome tends toward self-restraint, with $z^* = 1 - r$ and no mutual policing, $a^* = 0$. In B, when $r < 1 - c$, the outcome tends towards full investment in mutual policing and complete repression of competition, $a^* = 1$. In both A and B, z is shown on a log scale. In A, $c = r = 0.7$; in B, $c = 0.2$ and $r = 0.4$. The gradient of fitness with respect to mutual policing, $\partial w / \partial a$, is obtained by taking the partial derivative of w_{ij} with respect to a_{ij} , evaluated at fixed trait values for all individuals, $a_{ij} = a$ and $z_{ij} = z$. A similar approach is used for $\partial w / \partial z$. This is the standard method of evolutionarily stable strategy (ESS) analysis, as outlined by Maynard Smith¹⁹. An additional method for handling kin

selection is used here. The partial derivatives include the derivatives da_i/da_{ij} and dz_i/dz_{ij} . We show elsewhere (S.A.F. and P. D. Taylor, manuscript in preparation) that, by equating these derivatives with the slope of group genotype on individual genotype, these terms are simply the kin-selection coefficient of relatedness, r . This method has a close relationship to the Price equation²⁰, which has been used extensively for analysis of kin-selection problems²¹. Other assumptions about costs, benefits and genetics complicate the analysis. However, this minimal model demonstrates the powerful selective pressures on self-restraint and mutual policing that favoured increasingly complex evolutionary units.

As noted above, relatedness r is often less than one in complex groups. Thus mutual policing and suppression of competition are required for efficient social organization^{5,6,12}. The surprising result from a simple model is how strongly natural selection favours individual subunits to contribute resources for suppressing competition. Simultaneously, the subunits are still favoured to strive for their own success against their neighbours. Thus a double standard evolves. Individuals contribute toward universal fairness, but strive for their own reproductive gains within the system of fairness that they helped to create. This duality explains why components of a group, with their own self-interests, have contributed to complex regulatory mechanisms such as mutual policing of workers in insect societies¹² and mendelian segregation of chromosomes during meiosis. However, the dual interests of an individual explain why regulatory mechanisms are rarely sufficient to suppress individual striving within groups, and thus the rarity of the major evolutionary transitions to higher levels of organization.

At first glance, a likely scenario for the formation of higher-level units is the origin of complex regulatory mechanisms and policing when relatedness is high. Subsequent changes in social structure leading to lower relatedness within groups may follow once reproductive fairness is enforced. However, the model clearly shows that situations of high relatedness are the least conducive to large investments in regulatory mechanisms. Thus the origin of complex regulatory mechanisms cannot be explained solely by kin selection.

The model presented here clarifies the simple, logical relation between kin selection and the economics of regulatory mechanisms. The model in its simplest form is a theorem about natural selection rather than a testable prediction. The value of such abstract theory turns on its ability to unify apparently disparate phenomena by highlighting the fundamental processes common to each case. In addition, general understanding of process should lead to new insight for specific systems. A few examples illustrate the range of problems influenced by both kin selection and repression of competition.

Hurst¹³ showed that lower relatedness among cytoplasmic genetic elements tends to increase competition within the cell at a cost to individual fitness. This idea is the same as that expressed by equation (1), where lower relatedness leads to higher virulence. Hurst was interested in the forces that influence biparental versus uniparental inheritance of cytoplasmic elements. When there is cytoplasmic competition associated with low relatedness in cells then the nuclear genes suffer reduced fitness because of cytoplasmic 'virulence'. Thus biparental inheritance of organelles, which mixes lineages and lowers relatedness within cells, can reduce nuclear fitness when compared with uniparental inheritance. If relatedness and self-restraint among biparentally inherited cytoplasmic elements are sufficiently low then costly nuclear mechanisms can evolve to enforce uniparental inheritance. Such mechanisms repress competition by preventing the mixing of cytoplasmic lineages. Hurst reviewed evidence suggesting an interaction between costly nuclear control mechanisms and patterns of mixing among cytoplasmic lineages.

Wilson and Sober^{1,14} suggested that group competition can be repressed by a randomization mechanism such that all group members have an equal chance of obtaining limited resources or reproductive opportunity. One example is fair segregation of homologues in meiosis. The remarkable life cycle of the slime mould, *Dictyostelium discoideum*, may be another example (D. S. Wilson, personal communication). After a single-celled feeding stage, amoebae aggregate to form a slug¹⁵, which migrates without feeding, and eventually develops into a fruiting body with spores supported by stalk cells. The puzzle is why some cells sacrifice their own reproduction to support that of other cells. One obvious explanation is kin selection. Cells can gain by sacrificing their own reproduction to enhance the reproduction of highly related neighbours. That may indeed be the explanation, but with 10⁵ cells per slug there are opportunities for multiple lineages to mix or for mutants to arise that cheat by never developing into stalk cells.

Developmental studies of *Dictyostelium* have shown that cell fate (stalk or spore) is determined early in cell aggregation. The spatial distribution of cell types is apparently random when fate is determined^{16,18}. The prestalk cells then migrate to the front and the spore cells aggregate in the rear of the slug. The initial random distribution of cell types certainly fits with the idea that random determination of reproductive opportunity promotes group cooperation beyond the self-restraint favoured by kin selection. However, it is not clear how the group could police a mutant that always developed into a spore. Thus three issues for *Dictyostelium* development highlight the main points of the theory: kin selection and self-restraint among cells within a slug, whether a randomization mechanism that is difficult to cheat is possible, and the cost of such a mechanism.

Kin selection and repression of internal competition have each been discussed widely but, for the most part, separately. The theory and examples presented here show the important interaction between these fundamental processes in the evolution of cooperation and the formation of higher-level units. □

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A gene triggering flower formation in *Arabidopsis*

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IN *Arabidopsis*, the apical shoot meristem produces lateral meristems that develop into either shoots or flowers. The decision to form flowers instead of shoots is mediated by the action of floral-meristem-identity genes, such as *APETALA1* (*API*) and *LEAFY* (*LFY*), which specify meristem fate^{1–7}. Here we show that transgenic plants which constitutively express the *API* gene show transformations of apical and lateral shoots into flowers, and that these plants flower much earlier than wild-type plants. These results indicate that *API* alone can convert inflorescence shoot meristems into floral meristems, and that ectopic *API* expression can dramatically reduce the time to flowering.

API and *LFY* act redundantly to specify meristem fate and, because no single meristem-identity gene is absolutely necessary for floral meristems to arise, it was unclear if either of them would be sufficient to convert shoots into flowers. The *API* gene, which encodes a putative transcription factor with a MADS domain, is normally expressed in young flower primordia and is not expressed in inflorescence shoot meristems⁴. We generated transgenic plants that constitutively express *API* from the cauliflower mosaic virus 35S (CaMV35S) promoter⁸ to determine whether ectopic *API* expression could convert shoots into flowers. The most striking feature of the 35S-*API* transgenic plants (Fig. 1) is that the normally indeterminate shoot apex (Fig. 1a) prematurely terminates as a floral meristem and forms a terminal flower (Fig. 1e). In addition, all lateral meristems that would normally produce inflorescence shoots (Fig. 1b) are also converted into solitary flowers (Fig. 1f). These results demonstrate that *API* alone is sufficient to convert inflorescence shoots into flowers, even though *API* is not normally absolutely required to specify floral meristem identity. The conversion of shoots into flowers in the 35S-*API* transgenic plants is strikingly similar to the phenotypes caused by mutations in the *Arabidopsis* *TERMINAL FLOWER* (*TFL*) gene (Fig. 1c, d)^{9–11}. These observations suggest that the conversion of shoots into flowers by ectopic *API* expression may result from an inhibition of *TFL* activity.

To determine whether the 35S-*API* transgene causes ectopic *LFY* activity, and whether ectopic *LFY* activity is required for

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the conversion of inflorescence meristems into floral meristems, we introduced the 35S-AP1 transgene into *lfy* mutant plants. We found that *lfy* mutant plants containing the 35S-AP1 transgene display the same conversion of apical and lateral shoot meristems into floral meristems as was observed in wild-type plants (Fig. 1g, h). However, the resulting flowers have the typical *lfy* mutant phenotype, in which floral organs develop as sepaloid and carpelloid structures, with an absence of petals and stamens^{3,12,13}. Thus *LFY* is required to form the normal complement of floral organs in the 35S-AP1 transgenic plants but is not required for shoots to develop as flowers.

It has recently been shown that *LFY*, like *AP1*, is sufficient to convert shoots into flowers¹⁴. Mutations in the *AP1* gene

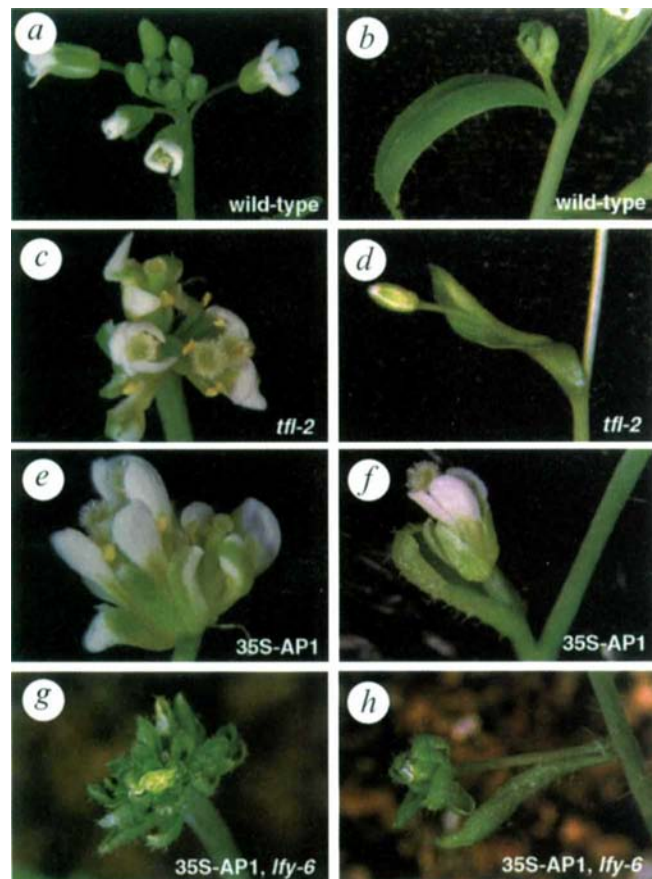


FIG. 1 Ectopic expression of *AP1* converts inflorescence shoots into flowers. Indeterminate shoot apex (a) and lateral shoot developing in the axil of cauline (stem) leaf (b) of wild-type *Arabidopsis* showing clusters of developing flowers. Shoot apex develops as a fused terminal flower (c) and lateral shoots develop as solitary flowers (d) in *tfl* mutant plants. 35S-AP1 transgenic plants are strikingly similar to *tfl* mutants, with apical (e) and lateral (f) shoots developing as flowers. When the 35S-AP1 transgene is introduced into *lfy-6* mutant plants, apical (g) and lateral (h) shoots develop as flowers. These flowers lack petals and stamens. The 35S-AP1 transgene fully rescued the severe *ap1-15* allele in the Columbia ecotype, indicating that this construct confers full *AP1* activity (not shown). All 35S-AP1 transgenic lines showed leaf curling, particularly in the young rosette leaves.

METHODS. *Bam*HI linkers were ligated to the *Hinc*II site of the full-length *AP1* complementary DNA⁷ in pAM116, and a resulting *Bam*HI fragment was fused to the cauliflower mosaic virus 35S promoter¹⁹ in pCGN18 to create pAM563. Kanamycin-resistant transgenic plants (T1) of the Columbia ecotype were selected after *Agrobacterium*-mediated plant transformation using the *in planta* method²⁰. All analyses were performed in subsequent generations. Approximately 120 independent transgenic lines that displayed the described phenotypes were obtained. The 35S-AP1 transgene was crossed into the strong *lfy-6* mutant background, and analyses done on the F₂ progeny.

significantly attenuate the phenotype of 35S-*LFY* transgenic plants, but the phenotype of the 35S-AP1 plants is not significantly attenuated by mutations in *LFY*. These data suggest that *AP1* acts downstream of *LFY* to specify meristem identity, and that one of the meristem-identity roles of *LFY* may be to activate *AP1*. Consistent with this idea is that the accumulation of *AP1* RNA in wild-type plants immediately follows the onset of *LFY* expression^{3,4,15}, and that the accumulation of *AP1* RNA is significantly delayed in *lfy* mutant plants (C. Gustafson-Brown and M.F.Y., unpublished results).

As well as being involved in the early step of specifying floral meristem identity, *AP1* is involved at a later stage in flower development by specifying sepal and petal identity^{16,17}. Molecular studies have shown that, although *AP1* RNA is initially expressed throughout the young flower primordium, it is later excluded from stamen and carpel primordia^{4,15}. However, because the cauliflower mosaic virus 35S promoter is normally active in all floral organs, and the 35S-AP1 transgenic plants have normal stamens and carpels, it is likely that *AP1* is not sufficient to specify organ fate.

The ectopic expression of *AP1* also influences the vegetative phase of plant growth, in addition to its ability to alter inflorescence meristem identity. Wild-type plants have a vegetative phase in which a basal rosette of leaves is produced before the transition to reproductive growth. Under continuous-light growth conditions, flowers are first visible on wild-type plants after approximately 18 days (Fig. 2a), whereas the 35S-AP1 transgenic plants flower after an average of only 10 days (Fig. 2b). Under short-day growth conditions, flowering is delayed in wild-type plants until approximately 10 weeks after germination (Fig. 2c), whereas the 35S-AP1 transgenic plants flower in less than 3 weeks (Fig. 2d). Thus ectopic *AP1* activity significantly reduces the time to flowering and reduces the delay to flowering caused by short-day growth conditions. Constitutive expression of several other MADS-box genes has also been shown to cause an

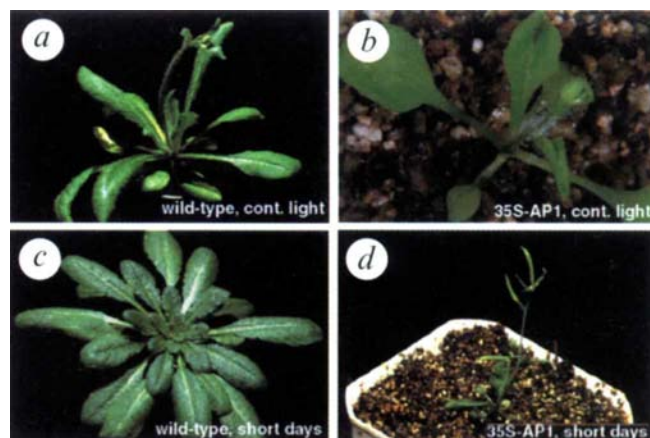


FIG. 2 Ectopic expression of *AP1* causes early flowering. 21-day-old wild-type (a) and 11-day-old 35S-AP1 transgenic (b) *Arabidopsis* plants grown under continuous light, shortly after the transition to flowering. Note the reduced vegetative phase of the transgenic plant indicated by the reduced rosette leaf number. Eight-week-old wild-type (c) and five-week-old 35S-AP1 transgenic (d) plants grown under short-day growth conditions. Note that the wild-type plant has produced numerous leaves and has not yet begun to flower (c), whereas the transgenic plant produced only a few leaves, has flowered, and is developing seeds (d). **METHODS.** The transition from vegetative to reproductive growth was measured both in terms of the number of days post-germination until the first visible flowers were observed, and by counting the number of leaves. Under continuous light, wild-type and 35S-AP1 transgenic plants flowered after producing 9.88 ± 1.45 and 4.16 ± 0.97 leaves, respectively. Under short-day growth conditions (8 h light: 16 h dark, 24 °C), wild-type and 35S-AP1 transgenic plants flowered after producing 52.42 ± 3.47 and 7.4 ± 1.18 leaves, respectively.

early flowering phenotype¹⁸ (B. Savidge, S. Rounsley and M.F.Y., unpublished results).

Because ectopic *API* activity reduces the vegetative phase of the life cycle of a plant, it is clearly influencing the behaviour of the vegetative meristem. However, ectopic expression of *API* alone is unable to convert the vegetative apical meristem into a flower immediately after germination. These data suggest that additional floral-promoting factors present in the inflorescence meristem are required for this conversion or, alternatively, that floral-inhibiting factors present in the vegetative meristem prevent this conversion. Whether or not the early flowering phenotype is a direct consequence of *API* acting at the vegetative shoot apex, or is an indirect result of altered plant growth and metabolism caused by the altered activity of downstream genes regulated by *API*, remains to be determined. However, the ability to control flowering time by ectopic *API* expression should prove a useful tool, not only for investigating the factors that regulate floral induction, but also for reducing the time to flowering of agriculturally important crop plants. □

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Requirement for *Drosophila* cytoplasmic tropomyosin in *oskar* mRNA localization

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THE localization of *oskar* (*osk*) RNA to the posterior pole of the developing fruit fly (*Drosophila*) oocyte induces the assembly of pole plasm, causing development of the abdomen and germ line^{1,2}. Failure to localize *oskar* RNA results in embryos that lack abdomen and germ cells. Conversely, mis-targeting of *oskar* RNA to the anterior of the oocyte causes formation of ectopic abdomen and germ cells at the anterior pole³. Maternal mutants that have reduced pole plasm activity produce sterile adults with normal abdominal development, suggesting that germ cells are more sensitive than abdomen to defects in pole plasm assembly⁴. Thus muta-

tions in genes that reduce *oskar* RNA localization or activity can be recovered as viable sterile adults. In a screen for mutants defective in germ cell formation, we isolated nine alleles of the tropomyosin II gene⁵. Here we show that mutations in *tropomyosin II* (*TmII*) virtually abolish *oskar* RNA localization to the posterior pole, suggesting an involvement of the actin network in *oskar* RNA localization.

Embryos derived from *TmII*^{gs1} females fail to form pole cells, the precursors of the germ cells (Fig. 1a, b), and develop into sterile adults. In the three molecularly characterized lines (*TmII*^{gs1}, *TmII*^{gs2} and *TmII*^{gs3}), the transposable element has inserted in the *TmII* gene, immediately downstream of the first exon of the cytoplasmic form of tropomyosin (cTm)^{5,6}. cTm is expressed exclusively in the ovary, where it is the major *TmII* isoform detected⁵. Northern analysis reveals that the level of cTm transcripts is reduced in *TmII*^{gs1} mutant ovaries (Fig. 1e).

To determine whether *TmII*^{gs1} mutants are sterile through a defect in pole plasm assembly or in pole cell formation *per se*, we assayed the ability of anteriorly localized *osk* RNA (ref. 3) to induce pole cells at the anterior pole in the *TmII*^{gs1} mutant

TABLE 1 Germline clone analysis of lethal excisions of *TmII*^{gs1}

Allele	Clones per female (n)	Eggs per female	Embryos with posterior group phenotype	Viable adult gs progeny
+	0.35 (450)	9.6	—	—
el3	0.14 (653)	2.7*	+	+
el4	0.16 (434)	4.3	+	+
er4	0.13 (508)	3.2*	+	+

Mosaic egg chambers composed of homozygous mutant germ cells and heterozygous follicle cells reproduce the germ-cell-less phenotype of the original *TmII*^{gs} mutants. Conversely, mosaic egg chambers produced by transplantation of wild-type pole cells into homozygous *TmII*^{gs} embryos result in wild-type egg chambers and wild-type embryos (J. Szabad, personal communication). Taken together, these results show that the *TmII* germ-cell-less phenotype is germline-dependent. The average number of clones per female was calculated by the Poisson distribution. We also observed a small but significant reduction in the number of eggs in the mutants, indicating a partial cell-lethal effect of the *TmII* lethal alleles in the germ line. The *TmII*^{gs} mutations were isolated as homozygous viable PLacZ (w⁺) insertions²². The transposon was mobilized from the X chromosome with a *trans* transposase source, the Δ2-3 element. The new autosomal insertion-bearing male individuals were selected for their w⁺ eye colour. Stable insertional lines were established with the *T(2,3)CyO* *l(2)513 Tm2 Ultrabithorax* (Ubx) translocated balancer chromosome. From each line, females homozygous for new PLacZ insertions were collected based on the absence of dominant markers of the translocated balancer chromosome. Germ-cell-less mutant lines were selected on the basis of sterility of the progeny of homozygous females. Analysis was carried out with *TmII*^{gs1}, *TmII*^{gs2} and *TmII*^{gs3}, from which the 5' and 3' genomic flanking regions of the inserted PLacZ elements were cloned by plasmid rescue²². A minimum of 150 base pairs (bp) of DNA flanking the PLacZ elements was sequenced using a primer derived from the P-element inverted repeat and bears 100% identity to sequences within the fourth exon and the 5' region of the fourth intron of the *TmII* gene. *TmII*^{gs1}, *TmII*^{gs2} and *TmII*^{gs3} are insertions at positions 3,753, 3,753 and 3,737 of the *TmII* genomic sequence²³, respectively. In *TmII*^{gs1}, the PLacZ element was remobilized, generating wild-type, grandchildless, as well as homozygous lethal excision alleles of the *TmII* gene. The lethal excisions *TmII*^{el3}, *TmII*^{el4} and *TmII*^{er4} were used to generate X-ray-induced homozygous germ-line clones. The *TmII*^{gs} insertions were mapped to the 88F chromosomal region. Df(3R)ea^{5022RX1}, an 88F overlapping deficiency, does not complement the *TmII*^{gs} mutations and the hemizygotes show a phenotype identical to that of homozygotes. Germline clones of lethal *TmII* alleles in *trans* to the dominant female sterile insertion *P[ovo^{D1}]* on chromosome 3R were induced by X-rays²⁴. *P[ovo^{D1}]/TmII* lethal heterozygotes were irradiated (1,000 rad, 150 rad min⁻¹, 150 kV) at 72±3 h after egg laying²⁵. The irradiated females were collected as virgins and mated with Oregon R (wild-type) males. Egg production of groups of 10 irradiated females were recorded over 12 days.

* $P < 0.05$; χ^2 -test.

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background. Embryos derived from *osk bicoid*; *TmII^{gs1}*/*TmII^{gs1}* females form pole cells at the anterior (Fig. 1c, d). This indicates that the *TmII^{gs1}* mutation does not affect germ cell formation *per se*. Rather, localization or maintenance of pole plasm molecules at the posterior pole is defective in *TmII^{gs1}* mutants. Indeed, when embryos from mutant females are greatly overstained with an antibody against the *osk* protein, only a trace amount of *osk* protein is observed, exclusively at the posterior pole (data not shown). Nonetheless, a gradient of *nanos* protein is formed (data not shown) and abdomen development is normal in 98% of embryos from *TmII^{gs1}* homozygous females, leading to viable but sterile adults.

To investigate whether stronger mutant alleles of *TmII* might have a more dramatic effect on oocyte development, we carried out germline clonal analysis with lethal alleles generated by excision of the P element from the original allele, *TmII^{gs1}*. As shown in Table 1, germ cells homozygous mutant for the lethal *TmII* alleles give rise to viable egg chambers that on fertilization develop into sterile adults, as well as to embryos that show abdominal defects. Hence, germline clones of lethal *TmII* mutations have a phenotype similar to that of *TmII^{gs1}*. This result suggests that *TmII* function is specific for pole plasm formation during oogenesis.

Localized *osk* RNA is required to induce assembly of a functional posterior pole plasm^{1,2}. During the early stages of oogenesis, *osk* RNA is enriched in the oocyte. At stage 8, *osk* RNA shows a transient anterior accumulation and the onset of posterior localization. By stage 9, *osk* RNA is present exclusively at the posterior pole. In *TmII^{gs1}* mutants, the initial enrichment of *osk* RNA in the oocyte is normal, but translocation of the RNA to the posterior pole fails (Fig. 2a, b). Instead, at stage 8, *osk* RNA accumulates at the anterior margin of the oocyte, where it remains until stage 10. The *staufer* gene (*stau*)⁷ is required for *osk* RNA localization, and in wild-type oocytes, RNA-binding protein STAU colocalizes with *osk* RNA. In the *TmII^{gs1}* mutants, STAU distribution precisely parallels that of *osk* RNA (Fig. 2c, d). The *TmII^{gs1}* defect seems to be specific to the posterior localization pathway, as the anterior localization of *bcd* and the anterior-dorsal localization of *gurken* (*grk*) are not perturbed (Fig. 2e-h).

The general importance of the cytoskeleton in RNA localization has been shown in a variety of organisms. Localized RNAs such as *Vg1* in *Xenopus* and *bcd orb*, *fs(1)K10*, *BicaudalD* and *osk* RNAs in *Drosophila* are enriched in detergent-resistant cytoskeletal fractions^{8,9}. *In vivo*, cytochalasin, an inhibitor of actin polymerization, prevents localization of *actin* mRNA to the per-

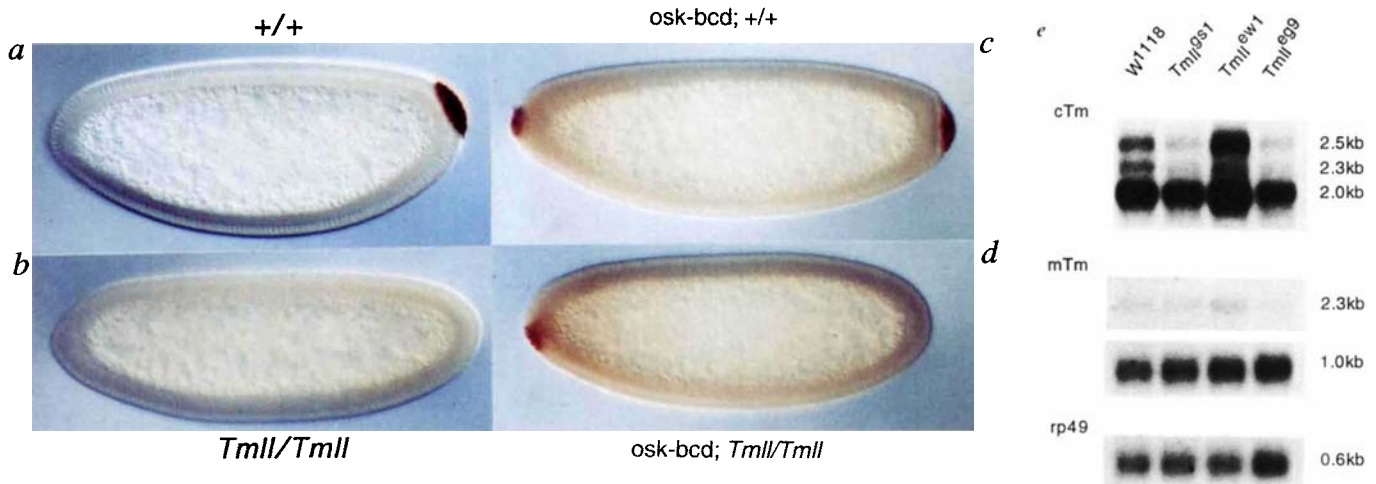


FIG. 1 Cytoplasmic tropomyosin is required for pole cell formation. a, b, Pole cells form at the posterior pole of wild-type embryos (a), but fail to form in embryos from *TmII^{gs1}*/*TmII^{gs1}* mutant females (b). c, d, Pole cells form at both poles in embryos from *osk-bcd3'UTR* females (c), but form exclusively at the anterior of embryos from *osk-bcd3'UTR*; *TmII^{gs1}*/*TmII^{gs1}* females. Embryos from *osk-bcd3'UTR* and *osk-bcd3'UTR*; *TmII^{gs1}*/*TmII^{gs1}* females form two abdomens of opposite polarity (data not shown). a–d, Pole cells are stained with an anti-NANOS antibody. a, b, Embryos at early cellular blastoderm stage; c–d, embryos at late syncytial blastoderm stage. All embryos orientated anterior left, dorsal up. The genotypes refer to that of the mothers from which the embryos are derived. e, cTm transcripts are reduced in *TmII* mutant ovaries. Northern blot bearing poly(A)⁺ RNA isolated from ovaries of *w¹¹¹⁸* (wild-type), *TmII^{gs1}* as well as *TmII^{ew1}* and *TmII^{eg9}*, two lines generated by excision of the P element from *TmII^{gs1}*. Mutants are described in Table 1 legend. *TmII^{eg9}* has the original gs phenotype of *TmII^{gs1}*, and *TmII^{ew1}* is a revertant to the wild type. When probed with a cTm-specific probe (upper panel) *TmII^{gs1}* and *TmII^{eg9}* reveal reduced levels of cTm transcripts, whereas the revertant *TmII^{ew1}* shows an increase in cTm transcripts to levels greater than *w¹¹¹⁸*. A muscle-specific probe (mTm) (middle panel) shows that the *TmII* mutations do not significantly affect the muscle transcripts detected. A probe specific for ribosomal protein 49 (rp49) (lower panel) serves as loading control. *TmII* is a complex gene, producing a large array of messenger RNAs by alternative splicing and differential polyadenylation. In addition, it is likely that several additional spliced transcripts exist that have not been identified or characterized²⁶. We have not yet determined the precise molecular

nature of the three (2.0, 2.3, and 2.5 kilobases (kb)) cTm transcripts, but presume they correspond to the 2.0-, 2.2- and 2.4-kb²⁶ and the 2.0-, 2.3- and 2.8-kb⁵ transcripts previously described, whose molecular nature is not fully understood. The 1.0-kb mTm transcript we detect using a probe specific for exons 1–3 may correspond to the 1.4-kb mRNA previously described⁵, or may represent an additional transcript. No 2.3-kb mTm mRNA has previously been reported, but this transcript is specific, as its amount increases in the revertant, and it is clearly distinct from cTm, because its profile differs from that of the cTm transcripts in the mutants.

METHODS. Whole-mount antibody staining of embryos was carried out as described elsewhere³. Nomarski optics. Poly(A)⁺ RNA (from 150 µg total RNA) was isolated from *w¹¹¹⁸* (wild-type), *TmII^{gs1}*, *TmII^{ew1}* and *TmII^{eg9}* mutant ovaries dissected in cold EB Ringer's, resolved on a 1.5% formaldehyde-agarose gel and blotted onto nitrocellulose. Hybridization was carried out at 55 °C in 50% formamide, 0.25 M NaPO₄ pH 7.2, 0.25 M NaCl, 1 mM EDTA, 7% SDS. cTm riboprobe: A *HindIII*–*XbaI* 0.55-kb fragment corresponding to exon 4 of *TmII* (bp 3,042–3,594) (ref. 23) was generated by polymerase chain reaction (PCR; information on primer sequences available on request) and subcloned into pSP72. Genomic DNA flanking the P element was isolated by plasmid rescue and served as template for the PCR. mTm riboprobe: A *HindIII*–*BglII* 0.31-kb fragment, covering exons 1–3 (bp 471–1,410; ref. 23), was generated by PCR from the mTm cDNA and subcloned into pSP72. rp49 riboprobe: A 0.42-kb PCR-generated fragment (407–571 bp²⁷) was subcloned into pBSK⁺. *In vitro* transcription of riboprobes was from the T7 promoter.

ipery in chicken fibroblasts¹⁰ and causes the release of localized *Vg1* mRNA from the vegetal pole of *Xenopus* oocytes¹¹. The microtubule drugs nocodazole, colchicine and taxol interfere with the translocation of *Vg1* RNA in *Xenopus*¹¹, and with localization of *bcd*, *orb*, *fs(1)K10*, *BicaudalD* and *osk* mRNAs in *Drosophila*^{12–14} oocytes, respectively. We therefore examined the organization of the cytoskeleton in *TmII^{gs1}* mutant ovaries. In *TmII^{gs1}* egg chambers, the filamentous actin network, revealed with rhodamine-conjugated phalloidin, seems to be normal (Fig. 3a, b). The distribution of microtubules also appears normal in *TmII^{gs1}* oocytes, as visualized with an anti- α -tubulin antiserum (data not shown). In addition, two microtubule motor proteins, dynein heavy chain (Dhc64C)¹⁵ (Fig. 3c, d) and a kinesin- β -galactosidase fusion protein¹³ (data not shown) are properly localized to the posterior pole of *TmII^{gs1}* oocytes. Ooplasmic streaming, a process that requires intact microtubules¹⁶, occurs in the mutant as in the wild type. As the actin microfilament and microtubule networks, the distribution of microtubule motor proteins and the process of ooplasmic streaming seem to be normal, we conclude that the overall integrity of the cytoskeleton is intact in *TmII^{gs1}*.

Our data show a role for cTm in *osk* RNA localization. Tropomyosin proteins are involved in actin-based motility in muscle cells, and cytoplasmic tropomyosin has been shown to associate with actin in non-muscle cells (reviewed in refs. 17, 18). In this context, our findings raise the possibility of an involvement of the actin cytoskeleton in RNA localization in the oocyte, a process in which microtubules have previously been implicated. We can envisage two ways in which cTm might function in this context. cTm protein might be an obligate component of a complex coupling *osk* RNA to putative microtubule-dependent motors that mediate its transport to the posterior pole. This model is supported by the observation that the *TmII^{gs1}* mutation uncouples the localization of *osk* RNA from that of the microtubule motor proteins dynein and kinesin- β -galactosidase. Alternatively, both actin and microtubule cytoskeletons might be required for *osk* RNA localization to the posterior pole. In support of this view, there is increasing evidence for functional interaction between the actin and microtubule cytoskeletal networks (for a review see ref. 19). For example, membranous organelles have been shown to move on both actin filaments and microtubules in squid axoplasm²⁰. In view of the possible involvement of

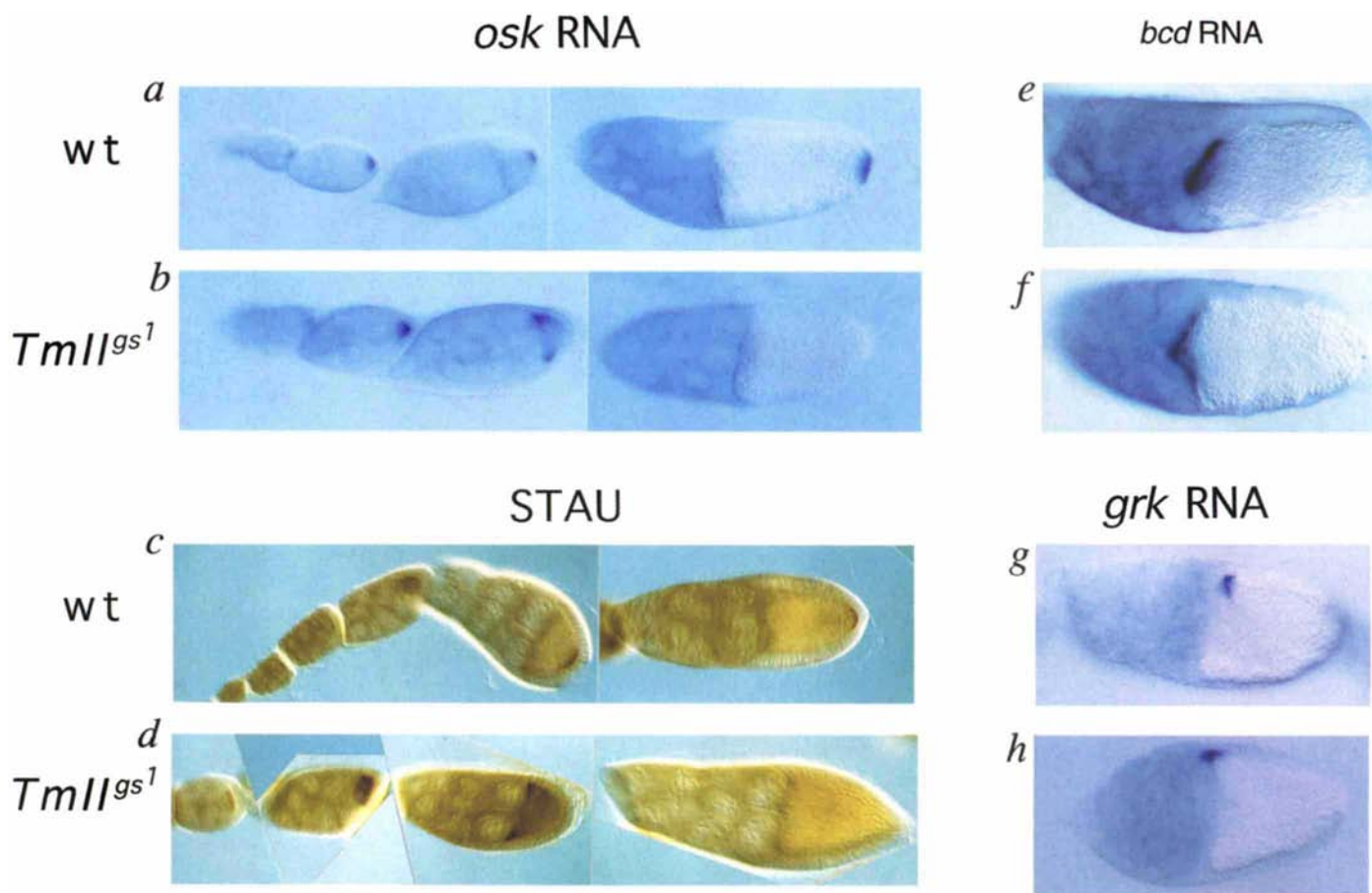
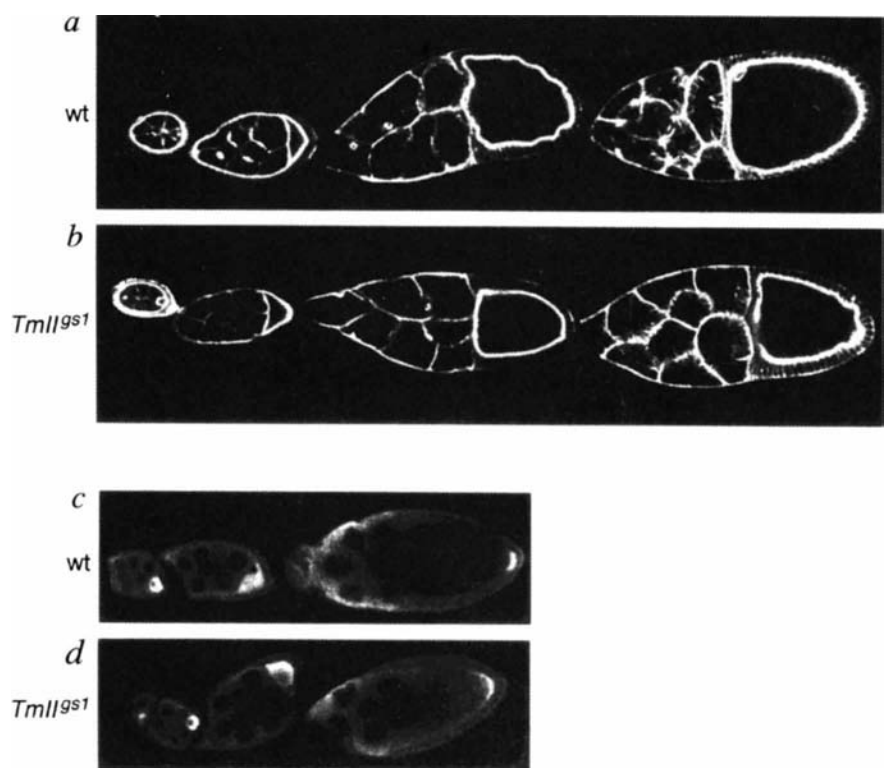


FIG. 2 *osk* RNA and *stau* protein fail to localize to the posterior pole in *TmII^{gs1}* oocytes. In wild-type (a, c, e, g) and *TmII^{gs1}* (b, d, f, h) ovaries, *osk* RNA (a, b) properly accumulates in the developing oocyte, but further localization to the posterior pole is altered in *TmII^{gs1}*. In the mutant, at stage 8 *osk* RNA accumulates at the anterior margin of the oocyte and by stage 10 of development is no longer detected. c, d, Distribution of STAU coincides with that of *osk* RNA. *bcd* (e, f) and *grk* (g, h) RNA distribution seems to be unaffected in *TmII^{gs1}*, as expected, as embryos produced by mutant females show normal head and thorax and dorso-ventral polarity. This indicates that cTm is only required for posterior localization or that its requirement in anterior and dorsal localization is far less important. Oocytes representing relevant stages of oogenesis were chosen from different

ovarioles to produce the composite ovarioles shown in these figures. METHODS. Digoxigenin-labelled DNA probes were used for *in situ* hybridization to ovaries, carried out as described¹. *osk* DNA probe corresponds to the 2.1-kb *SacI* fragment in the *osk* cDNA¹. *bcd* DNA probe corresponds to a 2.5-kb *EcoRI* fragment in *bcd*²⁸. *grk* DNA probe is a *XhoI*-*NotI* fragment from the *grk* cDNA²⁹. Whole-mount antibody stainings of ovaries were detected using biotinylated secondary antibodies and the Elite Kit (Vector Laboratories). After dissection, ovaries were fixed for 10 min in buffered 6% formaldehyde/heptane, permeabilized in methanol:DMSO (9:1), rehydrated into phosphate-buffered saline with 0.1% Triton X-100 (PBT), blocked in PBT/0.5% bovine serum albumin, and incubated with the anti-STAU antibody in blocking solution. Nomarski optics.

FIG. 3 Overall integrity of the cytoskeleton is intact in *Tmll^{gs1}* mutants. Wild-type (a) and *Tmll^{gs1}* (b) ovaries were stained with rhodamine-conjugated phalloidin to visualize filamentous actin. In both wild-type and mutant egg chambers, all three distinct populations of filamentous actin are observed: subcortical actin network in the oocyte, the nurse cells and the follicle cells; ring canals connecting the nurse cells and the oocyte, allowing intercellular transport through the cytoplasmic bridges; and actin filament bundles that assemble in the nurse cells of stage 10b egg chambers and are thought to hold the nurse cell nuclei in position during nurse cell contraction and the subsequent transfer of their cytoplasmic content into the oocyte. Stages from left to right: 6, 8, 10a and 10b. Wild-type (c) and *Tmll^{gs1}* (d) ovaries were stained with an anti-dynein (Dhc64C) antibody. In both wild-type and mutant, dynein protein is enriched in the oocyte and at stage 8 is localized to the posterior pole. a–d, Oocytes representing relevant stages of oogenesis were chosen from different ovarioles to produce the composite ovarioles shown.

METHODS. a, b, Ovaries were stained with rhodamine-conjugated phalloidin (Sigma)³⁰; c, d, Ovaries were stained with an anti-dynein (Dhc64C) antibody using a fluorescein-coupled secondary antibody. Images were obtained with the EMBL confocal scanning laser beam microscope (CCM)³¹.



actin, it will be interesting to test whether other actin-binding proteins such as profilin, villin and fascin, encoded by the *Drosophila* genes *chickadee*, *quail* and *singed* (for a review see ref. 21), are also involved in *osk* RNA localization. □

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Transient increase in *obese* gene expression after food intake or insulin administration

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OBESITY is a disorder of energy balance, indicating a chronic disequilibrium between energy intake and expenditure¹. Recently, the mouse *ob* gene², and subsequently its human and rat homologues^{2,6}, have been cloned. The *ob* gene product, leptin⁷, is expressed exclusively in adipose tissue, and appears to be a signalling factor regulating body-weight homeostasis and energy balance^{2,7–9}. Because the level of *ob* gene expression might indicate the size of the adipose depot, we suggest that it is regulated by factors modulating adipose tissue size. Here we show that *ob* gene exhibits diurnal variation, increasing during the night, after rats start eating. This variation was linked to changes in food intake, as fasting prevented the cyclic variation and decreased *ob* messenger RNA. Furthermore, refeeding fasted rats restored *ob* mRNA within 4 hours to levels of fed animals. A single insulin injection in fasted animals increased *ob* mRNA to levels of fed controls. Experiments to control glucose and insulin independently in animals, and studies in primary adipocytes, showed that insulin regulates *ob* gene expression directly in rats, regardless of its glucose-lowering effects. Whereas the *ob* gene product, leptin, has been shown to reduce food intake and increase energy expenditure^{7–9}, our data demonstrate that *ob* gene expression is increased after

food ingestion in rats, perhaps through a direct action of insulin on the adipocyte.

In C57BL 6J-*ob/ob* mice a nonsense mutation results in the production of truncated, non-functional leptin². Remarkably, *ob* mRNA is increased in these mice, suggesting that the absence of functional leptin activates regulatory mechanisms turning on *ob* gene expression. Similarly, in other animal models of obesity, such as C57BLKS-*db/db* mice¹⁰ and Zucker (*fa/fa*)⁵, and ventromedial hypothalamus-lesioned rats^{6,10}, *ob* mRNA levels are upregulated. These observations suggest that the level of *ob* gene expression indicates the size of the adipose depot in an attempt to maintain body-weight homeostasis, so *ob* gene expression should reflect the nutritional status of the animal. To test this hypothesis, we investigated whether changes in food intake modulate *ob* gene expression. Rats allowed food at night had adipose tissue *ob* mRNA levels that were twice those of animals fasted overnight (Fig. 1a, b). Because the onset of the night is associated with food intake in the rat¹¹, diurnal changes in adipose tissue *ob* mRNA levels were next measured in rats with free access to food. We found that *ob* mRNA levels were lowest during the light cycle, increasing soon after the rats start eating (20:00), reaching a maximum around 4:00 (Fig. 1c, d). Thereafter, *ob*

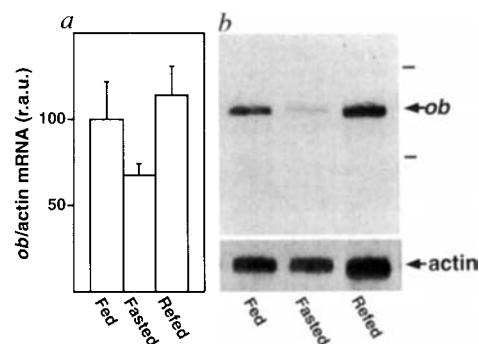


FIG. 2 The effects of acute food intake on *ob* mRNA levels in overnight-fasted rats. **a**, Levels of *ob*/actin mRNA. **b**, Northern blot of mRNA levels. METHODS. Rats were divided into 3 groups ($n=3$ each). A fed control group was allowed free access to food. Both other groups were denied access to food during the dark-light cycle. At the beginning of the light cycle, one group of fasted animals was given free access to food, whereas the other group served as a fasting control.

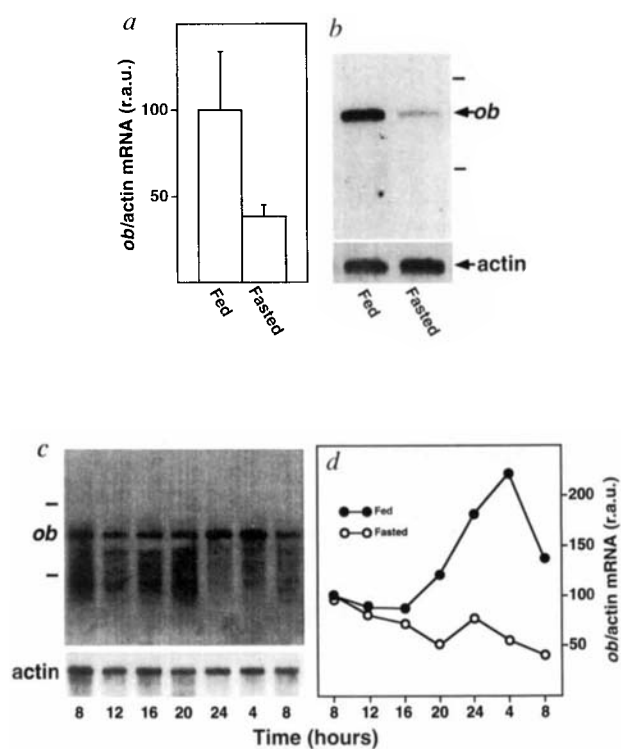


FIG. 1 Effect of fasting and diurnal rhythm on adipose tissue *ob* mRNA levels. **a**, Levels of *ob* mRNA, expressed as relative absorbance units (r.a.u.), in fasted and fed animals (mean $\pm$ s.d.). **b**, Northern blot of adipose tissue *ob* and γ -actin mRNA levels in an overnight fasted and fed animal. **c**, Northern blot showing diurnal variation of *ob* and γ -actin mRNA levels in rats with free access to food. **d**, Comparison of adipose tissue *ob* mRNA between rats fed (filled circles) or fasted (open circles) during the diurnal cycle.

METHODS. Adult male Sprague-Dawley rats were group-housed and accustomed to a 12:12 h light:dark cycle (light from 8:00 to 20:00). Adipose tissue *ob* mRNA levels were compared between fed and overnight fasted animals. ($n=3$ per group). To determine the diurnal variation of *ob* gene expression, a rat ($n=4$ per group) was killed every 4 h for 24 h. The influence of food intake on the diurnal variation was measured by comparing *ob* mRNA expression between fed rats and rats having no access to food during this period. Rats were killed, epididymal adipose tissue RNA was prepared and analysed by northern and dot-blot hybridization as described¹⁴. A mouse *ob*¹⁴ and a human γ -actin¹⁵ complementary DNA clone were used as probes.

mRNA steadily decreased, reaching a minimum in the afternoon (Fig. 1c, d). To study whether this diurnal rhythmicity was linked to changes in food intake, *ob* mRNA levels were next analysed in fasted animals over a 24-hour period. Compared to fed animals, the increase in *ob* mRNA during the night was completely abolished when food intake was prevented (Fig. 1d). Although diurnal variations in biological parameters are often correlated with cyclic changes in hormone levels and/or light, the absence of the cyclic variation of *ob* mRNA expression in fasted rats suggests that the observed surge in *ob* mRNA is a consequence of feeding, instead of being linked to a diurnal rhythm. We therefore speculate that food intake causes a rapid increase in *ob* mRNA levels.

To study the causal role of food intake on *ob* gene expression, animals were fasted overnight and refed at the beginning of the light cycle. Levels of *ob* mRNA in fasted rats were half those in fed rats. Levels of *ob* mRNA were restored to levels of normally fed animals 4 hours after ingestion (Fig. 2). Because feeding increases plasma insulin concentrations, we tested whether the effects of food intake on *ob* expression are mediated by insulin. Therefore, overnight fasted animals received a subcutaneous insulin injection (1 IU), and 4 hours later levels of *ob* mRNA were compared with those in fasted and refed animals (Fig. 3).

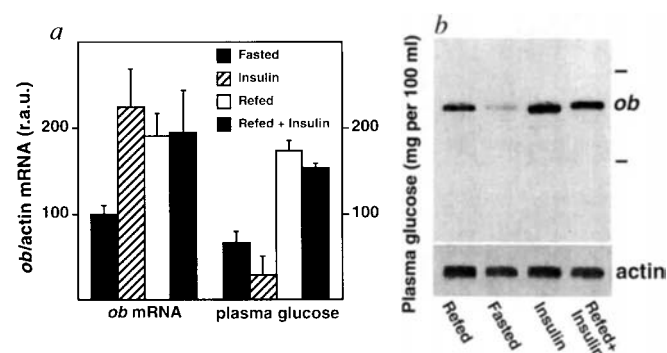
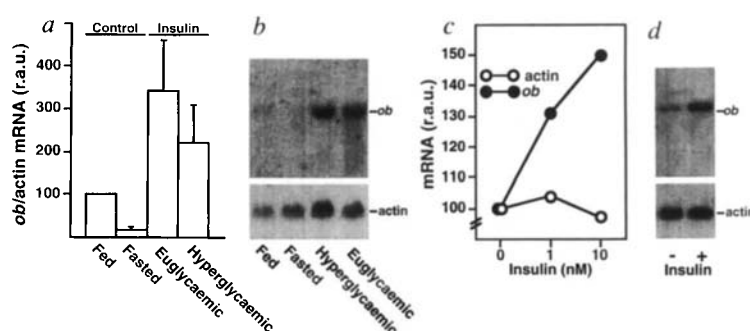


FIG. 3 Effects of insulin on *ob* mRNA levels *in vivo*. **a**, Effects of insulin, food intake, and insulin and food intake on *ob* mRNA levels in overnight-fasted rats. **b**, Representative northern blot.

METHODS. Overnight fasted rats were divided into 4 groups ($n=3$ each). One group served as a fasting control, and the other three groups received either free access to food, a single subcutaneous injection of insulin (1 IU Actrapid HMge, Novo-Nordisk), or food and insulin.

FIG. 4 Direct effect of insulin on *ob* mRNA expression. **a**, Graph depicting the effect of hyperglycaemic and euglycaemic hyperinsulinaemic clamps on *ob* mRNA. **b**, Northern blot comparing *ob* mRNA in rats subjected to hyperglycaemic and euglycaemic hyperinsulinaemic clamps to fasted and fed controls. **c**, Effect of different concentrations of insulin (1 and 10 nM) during 2 h on *ob* mRNA levels in primary rat adipocytes. **d**, Effect of 24 h exposure to insulin (100 nM) on *ob* mRNA expression in rat primary adipocytes. **METHODS**. Hyperglycaemic and euglycaemic hyperinsulinaemic clamps were performed during 6 h in Wistar rats as described¹⁶. Briefly, porcine insulin was infused at a constant rate of 10 nmol kg⁻¹ h⁻¹. Glucose levels were maintained at 96 ± 2 mg per 100 ml and 229 ± 4 mg per 100 ml in the euglycaemic and hyperglycaemic clamps, respectively, by a variable infusion of glucose. Glucose was determined every 5 min and insulin every hour during the clamp as described¹⁶. Adjustments of the glucose infusion were made to maintain glycaemia, as evident from the plasma glucose, and insulin levels: for the control (*n* = 7); 114 ± 10 mg per 100 ml and 46 ± 6 µU ml⁻¹; for the euglycaemic hyperinsulinaemic clamp (*n* = 3), 96 ± 2 mg per 100 ml and



670 ± 80 µU ml⁻¹; and for the hyperglycaemic hyperinsulinaemic clamp (*n* = 3), 229 ± 4 mg per 100 ml and 620 ± 70 µU ml⁻¹. At the end of the clamp, rats were killed and adipose tissue RNA was prepared as described. Primary rat adipocytes cultured as described¹⁷ were exposed to the indicated concentration of insulin or vehicle during 2 h (**c**) or 24 h (**d**). RNA was analysed as described above.

Insulin injection doubled *ob* mRNA levels compared to fasted controls so they were comparable to those in refed animals. Simultaneous injection with insulin and refeeding increased *ob* mRNA to a similar extent to insulin alone, indicating that insulin is sufficient to mimic the effects of food intake on *ob* expression. Because injection of insulin can be associated with counter-regulatory changes induced by the hypoglycaemic response to insulin, rats were rendered hyperinsulinaemic by subjecting them to both euglycaemic and hyperglycaemic hyperinsulinaemic clamps. Rats undergoing hyperinsulinaemic clamps, in which plasma glucose was respectively maintained at 96 ± 2 mg per 100 ml (slightly higher than fasting glucose levels, termed euglycaemic) or at 229 ± 4 mg per 100 ml (slightly higher than glucose levels after feeding, termed hyperglycaemic), showed a striking increase in *ob* mRNA levels relative to controls (Fig. 4a, b). These results demonstrate that the effect of insulin on *ob* gene expression is not due to changes in glucose concentrations. Finally, we investigated whether insulin affects *ob* mRNA expression in primary rat adipocytes. Consistent with the *in vivo* data, *ob* mRNA levels increased by up to 50% 2 hours after addition of insulin (1 and 10 nM; Fig. 4c). When primary adipocytes were exposed to a higher concentration of insulin during 24 hours, levels of *ob* mRNA were then doubled (Fig. 4d). This indicates that insulin has direct regulatory effects on adipocyte *ob* gene expression.

These data, together with the results of the diurnal variation and feeding studies, support a causal relationship between food intake, insulin levels, and *ob* gene expression. The close temporal relationship between these phenomena suggests that changes in feeding patterns regulate *ob* mRNA expression through an effect on insulin levels, which may explain the increased *ob* gene expression in hyperphagic hyperinsulinaemic animals, such as C57BL/6J-*ob/ob*² and C57BLKS-*db/db*¹⁰ mice, Zucker (*fa/fa*)⁵, or ventromedial hypothalamus-lesioned rats⁶. The reverse scenario, whereby *ob* controls food intake, is also probably true. Indeed,

late at night, when its levels are highest, *ob* probably acts as a satiety signal leading to a cessation of food intake and an increase in energy expenditure. Energy intake is tightly linked to energy expenditure by adaptive thermogenesis, which involves stimulation of heat production¹². Teleologically, this mechanism helps mammals to maintain body weight homeostasis by conserving energy during periods of food deprivation and preventing obesity during periods of energy excess. The *ob* gene product, leptin, may be an important mediator in this process⁷⁻⁹. An increase in leptin (as seen after eating) inhibits further food intake and/or regulates energy expenditure as part of a homeostatic mechanism to maintain body weight⁷⁻⁹. Decreased leptin levels in fasted animals might induce a sensation of hunger and, more importantly could be a stimulus for energy conservation, enabling them to survive periods of food shortage⁷⁻⁹. It is therefore conceivable that the level of *ob* expression is correlated with food intake, energy expenditure and the onset of obesity. Interestingly, hyperproinsulinaemia with diminished active insulin levels, owing to a mutation in the gene for carboxypeptidase E, was recently observed in obese C57BLKS-*fat/fat* mice¹³. Their diminished leptin levels might be due to the lower active insulin concentration, which might at least partly explain the obesity in these mice.

In conclusion, rat adipose tissue *ob* gene expression is regulated by feeding patterns, being reduced after fasting and increased after (re)feeding. Levels of *ob* mRNA are increased by insulin, indicating that insulin is an important mediator of the effects of food intake on *ob* expression. These data, together with our previous observation of the regulation of *ob* gene expression by glucocorticoids¹⁴, support the concept that *ob* gene expression is under hormonal control, which is expected for a key factor controlling body weight homeostasis and energy balance. Knowledge of the factors regulating *ob* gene expression should be of major importance in the prevention and treatment of obesity. □

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The role of neuropeptide Y in the antiobesity action of the obese gene product

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RECENTLY Zhang *et al.*¹ cloned a gene that is expressed only in adipose tissue of the mouse. The obese phenotype of the *ob/ob* mouse is linked to a mutation in the *obese* gene that results in expression of a truncated inactive protein. Human and rat homologues for this gene are known^{1,2}. Previous experiments^{3,4} predict such a hormone to have a hypothalamic target. Hypothalamic neuropeptide Y stimulates food intake, decreases thermogenesis, and increases plasma insulin and corticosterone levels making it a potential target⁵. Here we express the *obese* protein in *Escherichia coli* and find that it suppresses food intake and decreases body weight dramatically when administered to normal and *ob/ob* mice but not *db/db* (diabetic) mice, which are thought to lack the appropriate receptor. High-affinity binding was detected in the rat hypothalamus. One mechanism by which this protein regulated food intake and metabolism was inhibition of neuropeptide-Y synthesis and release.

Biosynthetic mature human and mouse *obese* gene product (hOB and mOB, respectively) with an intramolecular disulphide were prepared in *E. coli*, purified and tested for biological activity in rodents. Subcutaneous administration of hOB for 30 days to *ob/ob* mice led to a near normalization of body weight (Fig. 1a). The initial high dose of 300 µg led to a severe suppression of food intake (<25% of food consumed by *OB/?*; Fig. 1b) necessitating a dose reduction to 200 and 100 µg per mouse per day at days 9 and 11 to prevent malnutrition. Both lower doses maintained a similar suppression of food intake and promoted a loss of body weight throughout the 30-day period, indicating no downregulation. *db/db* mice, subjected to the same dose regimen, failed to respond (Fig. 1). Analysis of plasma glucose concentration at the end of the experiment demonstrated that the hyperglycaemic state was ameliorated (Table 1). Additionally, insulin levels were reduced to below the assay detection limit (similar to *OB/?* controls) and corticosterone levels were reduced significantly but not fully normalized.

The area of the arcuate nucleus that stained positive for prepro-NPY messenger RNA, by antisense ribonucleotide probe, was increased ($P < 0.05$, Fisher *post hoc* least significant difference test (PLSD); Fig. 2a) in *ob/ob* mice when compared with *OB/?* mice, as previously reported⁶. After the 30-day treatment with hOB, the number of neurons expressing neuropeptide Y (NPY) mRNA in this area was significantly decreased (Fig. 2a; $P < 0.05$). Such a reduction in NPY mRNA in the hypothalamus is consistent with a role for OB in the control of NPY biosynthesis. The greatest level of NPY mRNA expression was detected in the arcuate nucleus of *db/db* mice (Fig. 2a) and did not decrease ($P > 0.05$) after treatment.

Low doses of mOB (0.6–40 µg kg⁻¹) were sufficient to reduce normal dark-cycle feeding significantly during the first 24 hours when injected directly into the third ventricle (i.c.v.) of *ob/ob*

mice (Fig. 2b). Larger doses (400–4,000 µg kg⁻¹) were required to affect dark-cycle feeding significantly when administered subcutaneously (s.c.) to *ob/ob* mice (Fig. 2b). A further s.c. dose was required on the second day to obtain the same magnitude effect as that observed after a single i.c.v. injection. A faster and more potent anorectic action of OB when injected centrally supports the hypothalamic site of action.

To study effects of OB on the isolated hypothalamus, a region bordered dorsally by the thalamus, rostrally by the optic chiasm and caudally by the mammillary bodies was removed, bisected sagittally, washed and placed in a perfusion system. NPY was released in a peak during the first 2 hours of perfusion with medium containing 0.6 µM corticosterone. That glucocorticoid

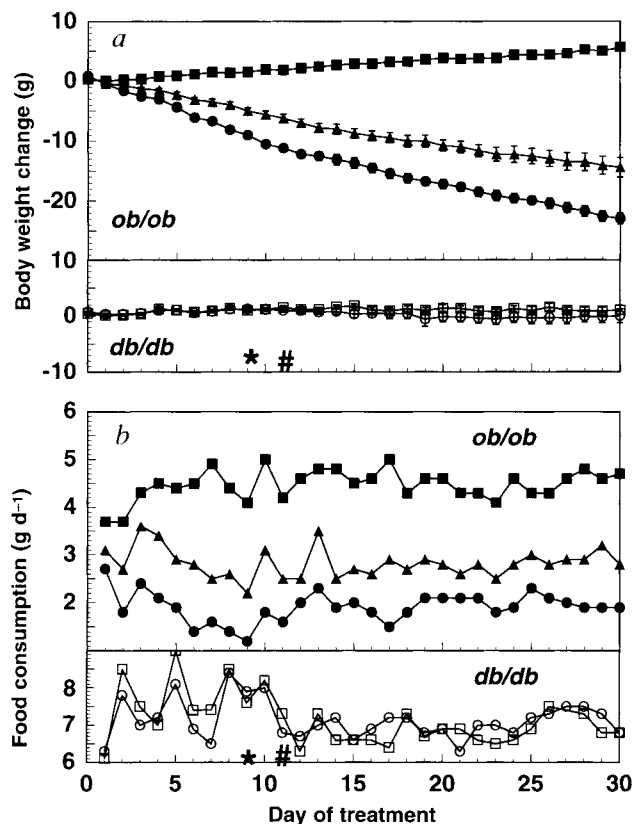


FIG. 1 Chronic effects of hOB on body weight change (a) were determined by administering the protein or saline subcutaneously daily 1 h before the dark cycle to *OB/?* (Δ), *ob/ob* (saline, $\blacksquare$; 300 µg, $\bullet$), and *db/db* (saline, $\square$; 300 µg, $\circ$) mice for 30 days. Blood samples were taken at the termination of the experiment. The high dose administered to *ob/ob* mice was 300 µg initially, but the excessive decrease in food consumption (b) led us to reduce the dose on day 9 to 200 µg (*) and again on day 11 to 100 µg (#) for the remainder of the study. *db/db* mice were given the same dosing regime. Body weight was measured before the first dose and at the same time on each subsequent day. Data are expressed as mean $\pm$ s.e.m. ($n=6$). All data points for *ob/ob* mice at both doses beginning from day 1 of the treatment are significantly different from controls ($P < 0.05$). Human *obese* cDNA was cloned by polymerase chain reaction after reverse transcriptase, sequenced, and subcloned into an expression plasmid. hOB expression in *E. coli* was at high level and formed inclusion bodies that were dissolved in 8 M urea. The protein was purified by anion-exchange chromatography. Peak fractions were diluted into phosphate buffer, repurified by size-exclusion chromatography and sterile filtered. Final protein was $\geq 95\%$ pure and endotoxin levels were ≤ 5 pyrogenic response units per mg protein. Experiments were done with 5–6-month-old male inbred lean (*OB/?*), and obese (C57Bk/6J-*ob/ob*) and (C57Bk/Ks-*db/db*) mice (Jackson Laboratories or Harlan). Both normal and diabetic mice were housed three or six per cage; water and Purina Formulab Chow 5008 (Purina Mills, St Louis, MO) were available *ad libitum*.

elevates NPY concentration has been demonstrated⁷. mOB inhibited cumulative NPY release during this period (204 ± 35 versus 130 ± 18 ng ml⁻¹ for control and 1 μ M mOB-treated, respectively, $P=0.048$). Low levels of NPY secretion observed without corticosterone stimulation prevented us from measuring OB inhibition of basal NPY release.

We also searched for specific binding of ¹²⁵I-labelled mOB (prepared using Bolton–Hunter reagent) to hypothalamic membranes. High-affinity binding ($IC_{50}=46$ nM, where IC_{50} is the 50% inhibitory concentration) and a dense population of receptors (9.4 ± 3.5 pmol per mg protein) were detected by competition binding studies (Fig. 3). Autoradiographic studies are being pursued to determine more specifically where the receptors are located in the hypothalamus and other portions of the brain.

Our studies demonstrate that OB regulates food intake and body weight in lean and genetically obese *ob/ob* mice. Chronic administration to *ob/ob* mice reversed obesity, hyperglycaemia,

TABLE 1 Effect of chronic s.c. hOB administration on blood chemistry in obese mice

	Glucose (mg dl ⁻¹)	Insulin (ng ml ⁻¹)	Triglycerides (mM)	Corticosterone (ng ml ⁻¹)
Obese mice				
<i>ob/ob</i> control	214 ± 16	75 ± 28	0.80 ± 0.07	247 ± 9
<i>ob/ob</i> low dose	$132 \pm 7^*$	$<2.3^{**}$	0.75 ± 0.04	$138 \pm 19^*$
<i>ob/ob</i> high dose	$118 \pm 7^*$	$<1.4^{**}$	0.66 ± 0.02	$95 \pm 18^*$
<i>OB/?</i>	$144 \pm 12^*$	$<1.5^{**}$	$1.00 \pm 0.07^*$	$31 \pm 6^*$
Diabetic mice				
<i>db/db</i> control	676 ± 18	$<1.4^{\dagger}$	2.00 ± 0.38	318 ± 17
<i>db/db</i> high dose	724 ± 18	$<1.5^{\dagger}$	1.49 ± 0.26	244 ± 32

See Fig. 2 for details. Data expressed as mean $\pm$ s.e.m. for $n=5$ or 6 mice per group with the exception of *ob/ob* high dose group where $n=4$ (one mouse with a urogenital infection was omitted). Plasma glucose concentration in mice was measured by a hexokinase method with a Monarch Clinical Autoanalyser (Instrumentation Laboratories). Plasma insulin was determined with radioimmunoassay kits from Diagnostic Products Corporation using rat insulin as standard. Plasma triglycerides of mice were measured with Sigma kit no. 336 (St Louis, MO). Corticosterone levels were analysed with a radioimmunoassay (Amersham).

* $P < 0.05$ compared with *ob/ob* control by t-test.

$\dagger$ Values are below the limit of detection.

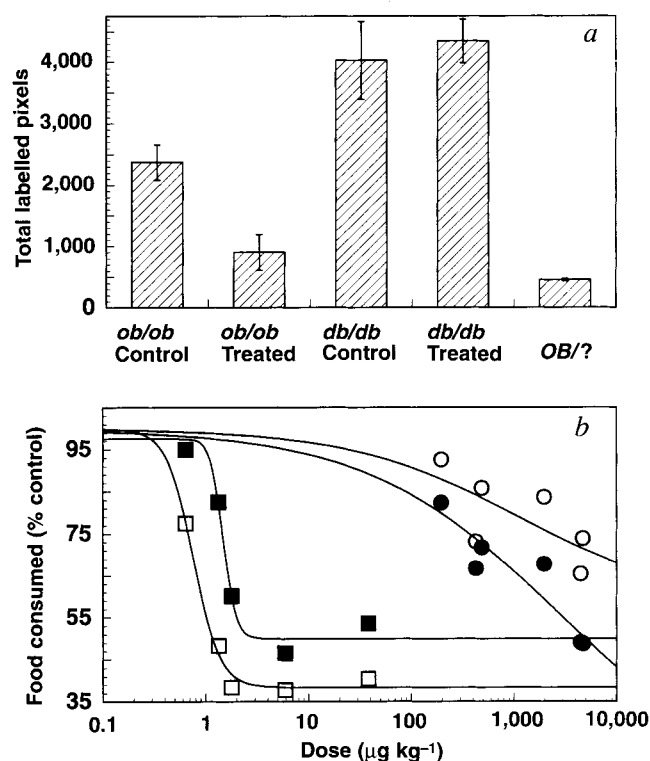


FIG. 2 a, Chronic effects of hOB on hypothalamic NPY levels (see Fig. 1 legend for details of treatment). Data are presented as mean total antisense pre-pro-NPY mRNA stained pixels per arcuate nucleus area $\pm$ s.e.m. ($n=4$). Animals were killed by decapitation, brains removed and fixed immediately. NPY was identified by hybridization with an antisense digoxigenin-labelled RNA probe. Antidigoxigenin antiserum, alkaline phosphatase and chromagen were used to colour the NPY mRNA-antisense probe hybrids. The riboprobe was the antisense sequence to the 288 bases of NPY pre-pro-mRNA overlapping the 5' and 3' non-coding region. Densitometric measurements of sections ($\sim 180 \mu$ m) were obtained with NIH Image 1.55. The feature outline of the arcuate nucleus was obtained manually by tracing directly on the video screen. b, Comparison of i.c.v. ($\square$, $\blacksquare$) and s.c. ($\circ$, $\bullet$) administration of mOB on normal dark-cycle feeding during day 1 ($\square$, $\circ$) and the following day ($\blacksquare$, $\bullet$) after first injection. mOB was administered either via the dorsal third ventricle or subcutaneously to *ob/ob* mice just before the onset of the dark cycle, and food consumption was measured during two consecutive 24-h periods. A second administration was given for subcutaneous treated mice on day 2. Data are from three separate studies for each parenteral route and are expressed as mean food consumed per animal as a percentage of vehicle control ($n=5-6$). I.c.v. cannulas were placed 1.5 mm lateral and 1.0 mm posterior to the bregma at a depth of 2.5 mm, and 1.0 μ l of mOB contained in Plasma-lyte were injected.

hyperinsulinaemia and hypercorticotonaemia. Induction of obesity by lesions of specific nuclei in the hypothalamus focuses attention on this site as a target for the adipose hormone⁸. Indeed, we observed a more potent anorectic action of OB administered centrally than when injected parentally. These data are the first to demonstrate high-affinity OB receptors in rat hypothalamus, but further studies on the distribution of the receptor are needed. We suggest that this receptor locus is the arcuate nucleus because it is outside the blood-brain barrier and because we observed that hypothalamic NPY mRNA expression was decreased by chronic OB treatment. Further, our data demonstrate that OB directly suppressed NPY release from isolated perfused normal rat hypothalami. Inhibition of NPY biosynthesis and release by the hypothalamus is consistent with decreased food intake, decreased plasma insulin and lower corticosterone levels⁵. These studies suggest that OB may be useful in treatment

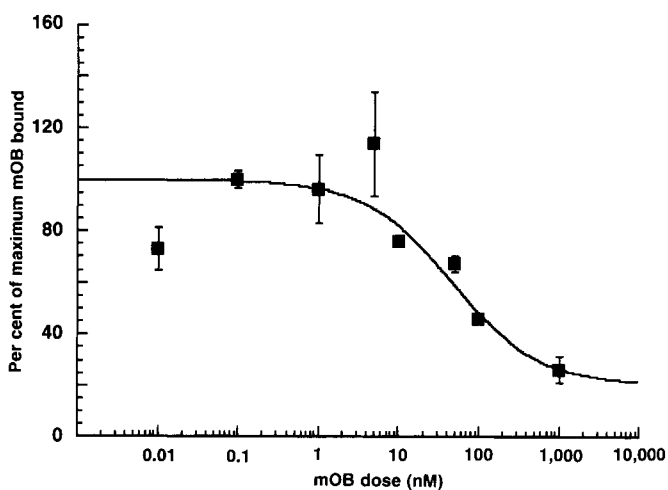


FIG. 3 High-affinity binding of mOB to rat hypothalamic plasma membranes. Data are expressed as the percentage of the maximum bound ¹²⁵I-labelled mOB $\pm$ s.e.m. Incubations were performed for 3 h at room temperature (160 μ g of hypothalamic membrane protein, ¹²⁵I-labelled mOB, unlabelled mOB from 0 to 1 μ M, 84.7 mM NaCl, 30 mM KCl, 1.2 mM MgSO₄ · 7H₂O, 11.2 mM NaH₂PO₄, 15 mM HEPES, 5.5 mM glucose, 50 μ g ml⁻¹ Bacitracin, 0.1% (w/v) BSA, pH 7.5). Bound fraction was collected by filtration, transferred to test tubes and counted for γ emission. Hypothalamic membranes were isolated by differential centrifugation from whole hypothalamic homogenates. mOB was iodinated by the Bolton–Hunter method¹².

of obesity when caused by an insufficiency or deficiency of the adipose hormone. Finally, during the revision process, three reports were published⁹⁻¹¹, also demonstrating the antiobesity effects of OB in *ob ob* mice. □

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Isolation and structure of the endogenous agonist of opioid receptor-like ORL₁ receptor

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THE ORL₁ receptor, an orphan receptor whose human¹ and murine²⁻⁸ complementary DNAs have recently been characterized, structurally resembles opioid receptors and is negatively coupled with adenylate cyclase¹. ORL₁ transcripts are particularly abundant in the central nervous system. Here we report the isolation, on the basis of its ability to inhibit the cyclase in a stable recombinant CHO(ORL₁⁺) cell line, of a neuropeptide that resembles dynorphin A⁹ and whose amino acid sequence is Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln. A rat-brain cDNA encodes the peptide flanked by Lys-Arg proteolytic cleavage motifs. The synthetic heptadecapeptide potently inhibits adenylate cyclase in CHO(ORL₁⁺) cells in culture and induces hyperalgesia when administered intracerebroventricularly to mice. Taken together, these data indicate that the newly discovered heptadecapeptide is an endogenous agonist of the ORL₁ receptor and that it may be endowed with pro-nociceptive properties.

Human¹ and murine²⁻⁸ cDNAs have been identified that encode ORL₁ (Opioid Receptor-Like 1), a new G-protein-coupled receptor whose amino acid sequence is most closely related to those of opioid receptors. The orphan receptor medi-

ates inhibition of forskolin-induced accumulation of cAMP by the opiate etorphine in a stable recombinant CHO(ORL₁⁺) cell line¹. This effect is exerted neither by the endogenous opioid peptides β -endorphin, enkephalins and dynorphins, nor by selective μ -, δ - and κ -opioid agonists^{1,5,6}. Furthermore, etorphine is about three orders of magnitude less potent in inhibiting the cyclase by means of the ORL₁ than by an opioid receptor.

ORL₁ transcripts are present in the mouse and rat central nervous system (CNS)^{1-6,8}, as well as in a few peripheral organs such as intestine, vas deferens and spleen⁵. In the CNS these transcripts are most abundant in limbic areas, hypothalamus, pons and spinal cord, suggesting that ORL₁ plays a role in many central processes, including learning and memory, attention and emotions, homeostasis and sensory perception. The potential widespread importance of ORL₁ in neurophysiology and, possibly, neurophysiopathology, led us to identify an endogenous ligand of this receptor.

The structural homology of ORL₁ with opioid receptors, in particular the acidic extracellular loop 2 which, in the case of the κ -opioid receptor, is required for high-affinity binding of dynorphins^{10,11}, suggested that the ligand might be a peptide that resembled a dynorphin. Therefore, we used an extraction procedure previously used¹² to isolate a pituitary peptide that was later identified as dynorphin A (ref. 9). Because the orphan ORL₁ receptor is negatively coupled with adenylate cyclase, the desired compound was expected to inhibit forskolin-induced accumulation of cAMP in the recombinant CHO(ORL₁⁺) but not in the non-recombinant CHO(ORL₁⁻) cell line, as previously shown for etorphine¹.

Size-exclusion chromatography of the crude peptide extract on Bio-Gel P-2 proved efficient in revealing the desired activity (Fig. 1a). The active fractions were recovered in the void volume (pool F1) and, to some extent, in pool F2 (not shown), indicating that the biologically active substance had a relative molecular mass (M_r) of around 1,800, which is the nominal exclusion limit of Bio-Gel P-2. The other pools were either inactive or equally effective in inhibiting (or stimulating in the case of F7) adenylate cyclase in the two CHO cell lines (data not shown). Pool F1 was further purified by cation-exchange fast protein liquid chromatography (FPLC) (Fig. 1b). Activity was recorded in consecutive 1-ml fractions eluted at about 0.4 M NaCl, indicating the strongly basic nature of the active compound(s). These two fractions were then applied directly onto a reversed-phase high-performance liquid chromatography (HPLC) column and gradient-eluted with acetonitrile (Fig. 1c) to yield enough material of sufficient purity for protein sequencing. This material was found to be a heptadecapeptide of average M_r 1,810 whose sequence was determined to be Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln. This most closely resembles dynorphin A (Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln)⁹, and may therefore be anticipated to interact with the ORL₁ receptor as the latter does with the κ -opioid receptor^{10,11,13}. In particular, it can be viewed as being made up of an amino-terminal Phe-Gly-Gly-Phe 'message' for biological activity, followed by a Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys 'address' for enhanced potency¹³. The 'address' contains all the basic amino acid residues that are anticipated to bind the acidic second exofacial loop of the ORL₁ receptor.

To ensure that the sequence in question was not that of a major contaminant, the heptadecapeptide was synthesized and the synthetic heptadecapeptide was found to be a potent inhibitor of forskolin-induced accumulation of cAMP in the recombinant CHO(ORL₁⁺) cell line (Fig. 2). Its 50% inhibitory concentration (IC₅₀) was 0.9×10^{-9} mol l⁻¹ and maximal inhibition was 90%. The synthetic peptide had no action on cyclase in the non-recombinant CHO(ORL₁⁻) cell line and displayed only micromolar or less potency in opioid receptor-mediated cyclase assays (data not shown).

The synthetic heptadecapeptide was active *in vivo* and affected reactivity to pain in the opposite manner to inhibition of ORL₁

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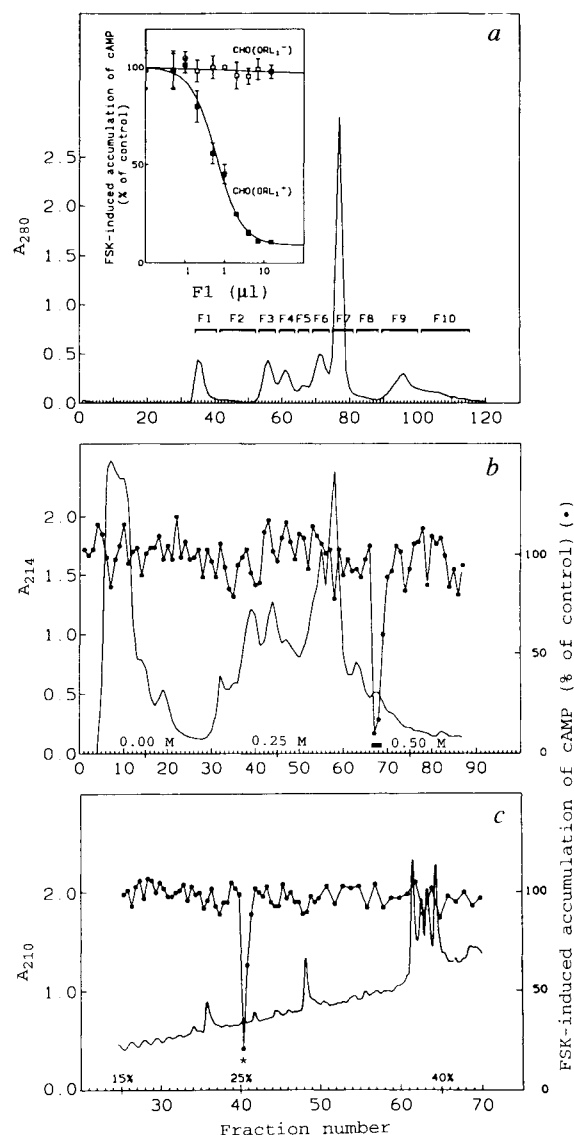
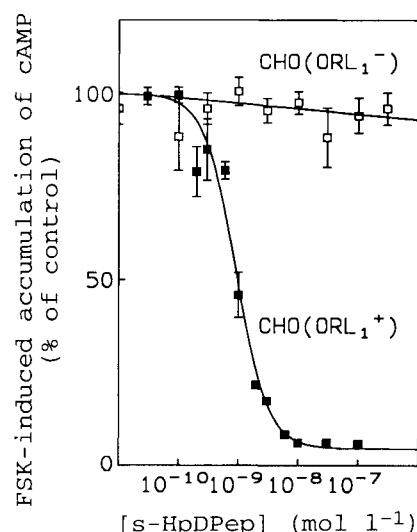


FIG. 1 Purification of the endogenous ligand of orphan receptor ORL₁. **a**, Size-exclusion chromatography of the crude peptide extract from rat brain. The insert shows that the sought-after activity, inhibition of FSK-induced synthesis of cAMP in CHO(ORL₁⁺) but not in CHO(ORL₁⁻) cells, is present in pool F1. **b**, Cation exchange FPLC of pool F1. The molarities above the x-axis are those of NaCl in the elution gradient. A major peak of activity was eluted at about 0.4 M NaCl (fractions 67 and 68, filled rectangles). **c**, Reversed-phase HPLC. The percentages above the x-axis are those of acetonitrile in the elution gradient. The desired biological activity, marked by an asterisk, was recovered as a single fraction which was eluted at 26% CH₃CN. FSK-induced accumulation of cAMP is shown (**b** and **c**) using filled circles.

METHODS. **a**, A crude extract¹² from freshly dissected and rapidly frozen rat brains (35 g) was lyophilized, dissolved in 10 ml 0.1 M AcOH and loaded onto pre-equilibrated Bio-Gel P-2 (2.3 × 90 cm column) at room temperature. Elution was at 0.6 ml min⁻¹. Fractions of 4 ml were collected, assayed for absorption at 280 nm and combined into pools F1–F10. Individual pools were lyophilized, dissolved in 2 ml phosphate buffer (20 mM NaPO₄, pH 6.4) and assayed for inhibition of forskolin-induced intracellular accumulation of cAMP in the CHO(ORL₁⁺) and CHO(ORL₁⁻) cell lines^{1,17}. The CHO(ORL₁⁺) line used here is a more responsive subclone of the original recombinant clone¹. Each data point is the mean ± s.e.m. of triplicate determinations. **b**, Pool F1 (1.8 ml) was applied to a SP/8HR cation exchange column (Waters) equilibrated with 20 mM NaPO₄ buffer, pH 6.4 (buffer A). Elution was at 1 ml min⁻¹ with 100% buffer A for 15 min followed by a linear gradient from 100% buffer A to 50% buffer B (20 mM NaPO₄ + 1.0 M NaCl, pH 6.4) in 60 min. Each fraction (10 µl) was assayed for cyclase inhibitory activity. **c**, The active fractions (67 and 68) were pooled and loaded directly onto a reversed-phase HPLC column (µ Bondapak C₁₈, 3.9 × 300 mm; Waters). The eluents were 0.1% TFA in H₂O (eluent A) and 0.1% TFA in acetonitrile (eluent B). Elution was at a flow rate of 1 ml min⁻¹ for 10 min with 100% A followed by a gradient to 10% B in 5 min and a gradient between 10% and 40% B in 50 min. Fractions of 0.5 ml were collected. Each fraction (10 µl) was dried (Speed Vac), redissolved in phosphate buffer and assayed for inhibition of cyclase in CHO(ORL₁⁺) cells. The molecular mass and sequence of the active compound in fraction 40.5 were determined with a Lasermat mass spectrometer (Finnigan MAT) and a model 476A protein sequencer (Applied Biosystems), respectively.

FIG. 2 Inhibition by the synthetic heptadecapeptide (s-HpDPep) of forskolin-induced accumulation of cAMP in recombinant CHO(ORL₁⁺) and non-recombinant CHO(ORL₁⁻) cells. Assay for cAMP in intact cells was as in the legend to Fig. 1. Each data point is the mean ± s.e.m. of triplicate determinations.



receptor expression (Fig. 3). In a hot-plate test¹⁴, mice that had been repeatedly treated with mAS[25,9], an antisense oligonucleotide¹⁵ to ORL₁ mRNA, displayed substantially increased latencies to rearing and escape jumping in comparison with saline-treated animals: 38 ± 2 s ($P < 0.001$) versus 20 ± 2 s, and 108 ± 6 ($P < 0.001$) versus 71 ± 6 s, respectively (Fig. 3a). The 'missense' oligonucleotide hAS[25,9], the human counterpart of mAS[25,9], was totally ineffective in this respect. In another set of experiments, mice were intracerebroventricularly injected with 10 ng (5.5 pmol) or 100 ng (55 pmol) of the new peptide (Fig. 3b). At the larger dose of peptide, the animals showed significantly decreased latencies to rearing and escape jumping: 14 ± 2 s (-36% , $P < 0.01$) versus 22 ± 2 s, and 48 ± 2 (-26% , $P < 0.001$) versus 65 ± 3 s, respectively. In other words, the animals had become hyperreactive to nociceptive stimulation.

Final evidence that the new peptide must be physiologically significant and synthesized locally was provided by the isolation, from a rat-brain library, of a cDNA of 0.925 kb whose deduced amino acid sequence contains that of the heptadecapeptide (Fig. 4). The complete messenger RNA, estimated by northern blot analysis to be in the range 1.2–1.3 kb, is only slightly longer than the cDNA. Within the precursor, the heptadecapeptide sequence is flanked by Lys-Arg proteolytic excision motifs¹⁶. The precursor polypeptide contains additional pairs of basic amino acid residues that delineate other putative biologically active peptides, one of 35 amino acids immediately upstream and another of 17 amino acids immediately downstream of the heptadecapeptide. In this respect, the peptide's precursor would be more similar to pro-opiomelanocortin which is the precursor of several unrelated bioactive peptides than pro-enkephalin and pro-dynorphin, which each contain several copies of closely related (opioid) peptides¹⁶.

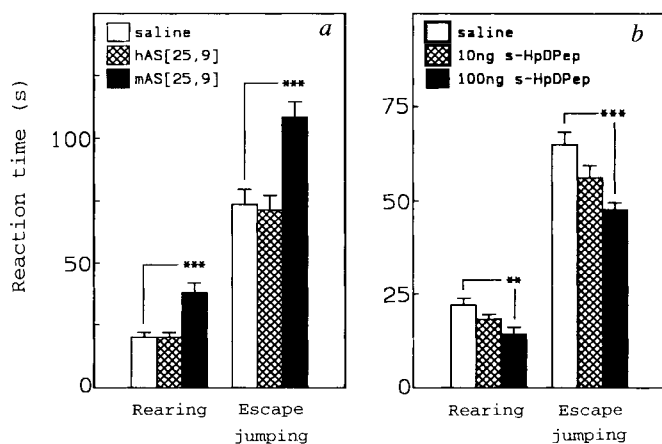


FIG. 3 Effects of repeated intracerebroventricular injection of antisense oligodeoxynucleotide (a) or a single injection of synthetic heptadecapeptide (b) on the latencies to rearing and escape jumping of mice in the hot-plate test¹⁴. Each bar represents the average reaction time $\pm$ s.e.m. from 20 animals. Statistical comparisons of oligo- or peptide- and saline (control)-treated groups were performed using the Student's *t* test. ** $P < 0.01$, *** $P < 0.001$.

METHODS. Male Swiss CD1 mice (20–25 g; Charles River) were used. a, Antisense oligonucleotide mAS[25,9] was the 17-mer 3'-GGAG-AAAGGACGGGGTA-5' complementary to bases 9–25 of the translated region of the mouse ORL₁ mRNA. Control missense oligonucleotide hAS[25,9], was the 17-mer 3'-GGAGAAGGGGCGCGCA-5' complementary to bases 9–25 of the translated region of the human ORL₁ mRNA. The oligonucleotides were dissolved in sterile physiological saline (0.9 g l^{-1} NaCl) at the final concentration of 2 mg ml^{-1} . The animals were manually injected with $10 \mu\text{l}$ of solution directly into the lateral brain ventricle every day for 4 consecutive days, and tested individually 96 h after the last injection. b, The animals were manually injected with 0 (control), 10 or 100 ng s-HpDPep in $10 \mu\text{l}$ of saline solution (9 g l^{-1} NaCl) and tested 20 min later. The experiment was repeated twice with essentially the same results.

In summary, three important observations lend support to the claim that we have identified the naturally occurring agonist of receptor ORL₁: (1) a synthetic peptide of identical structure to the natural peptide exhibits nanomolar potency in the ORL₁-mediated cyclase inhibition assay; (2) the natural peptide shares structural similarity with other known peptides, in particular dynorphin A; and (3) the sequence of the natural peptide is present, framed by Lys-Arg proteolytic excision motifs, in a large



FIG. 4 Partial nucleotide and deduced amino acid sequences of the rat cDNA encoding the precursor of the novel brain peptide. The pairs of basic amino acid residues which are presumably implicated in enzymatic processing of the propeptide are boxed. The sequence of the new peptide is surrounded by two such motifs and underlined. The sequences of two other putative bioactive peptides are indicated by a broken line. The asterisk marks the cDNA's stop codon.

METHODS. Degenerate oligonucleotides were designed on the basis of the amino acid sequence of the new peptide, taking into account the frequency of codon usage in mammals. Direct primer was AGATCTA-GACTT(CT)GG(CG)GG(CG)TT(CT)AC(ACGT)GG and reverse primer was CTTAAGCTT(CT)TG(AG)TT(ACGT)GC(CG)AG(CT)TT, corresponding to sequences 1–6 and 13–17 of the peptide, respectively. These oligonucleotides were used to amplify and clone by PCR on human, rat and mouse genomic DNA, the gene fragment encoding the peptide. The deduced central amino acids of the peptide (ARKSAR) were identical in the three species. The rat clone was used as a probe to screen a rat-brain cDNA library (Stratagen) constructed in lambdaZAPII. A single cDNA clone was isolated and sequenced.

precursor whose mRNA is expressed in the brain. The new peptide, whose sequence appears to be strictly conserved across mammalian species (ox (not shown), mouse, rat and man) (see legend to figure 4), must be of widespread importance. Because its first demonstrated physiological action appears to be increased reactivity to pain in intact animals, the new peptide might be named nociceptin. □

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Negative regulation of T-cell adhesion and activation by CD43

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CD43 is a cell-surface sialoglycoprotein expressed by a variety of haematopoietically derived cells, including T lymphocytes^{1–9}. Earlier observations of defective CD43 expression by T lymphocytes from boys with the X-chromosome-linked Wiskott-Aldrich syndrome suggested the importance of CD43 in lymphocyte function^{10,11}. Subsequent studies have suggested that CD43 facilitates leukocyte adhesion^{12–14} and has a co-stimulatory role during T-cell activation¹⁵. To define the physiologically relevant function(s) of CD43, we have generated CD43-knockout mice. We report here that CD43-deficient T cells from such mice show a marked increase in their *in vitro* proliferative response to concanavalin A, anti-CD3, the superantigen SEB and allostimulation. Additionally, CD43-deficient T cells show a substantial enhancement in homotypic adhesion and in their ability to bind different ligands, including fibronectin and the intercellular adhesion molecule ICAM-1. Vaccinia-virus-infected CD43-knockout mice mounted an augmented anti-vaccinia cytotoxic T-cell response compared with their wild-type littermates, yet developed an increased virus load. We conclude that CD43 negatively regulates T-cell activation and adhesion and is important for viral clearance.

CD43-knockout mice were generated by homologous recombination via embryonic stem-cell chimaeras (Fig. 1). Although CD43 is expressed from a very early stage in haematopoiesis^{8,9} and the pattern of expression varies in a lineage-specific

manner^{16,19}, no developmental defect was observed in the mutant mice. The mice develop and breed normally. Haematocrit values were normal, as were blood leukocyte and platelet counts. Histology of bone marrow, thymus, spleen and lymph nodes from CD43-knockout mice resembled that of wild-type mice. Flow-cytometric analysis of thymocytes and splenocytes revealed no gross immunophenotypic abnormalities other than the loss of CD43 expression. T-lymphocyte numbers, CD4 and CD8 subset proportions, levels of CD3, CD4 and CD8 expression, and B-cell (B220⁺, IgM⁺) numbers in spleens from CD43-knockout mice were comparable to those of their wild-type littermates. T-lymphocyte expression of activation markers, including interleukin-2 α chain, Ia antigen and lymphocyte function-associated antigen-1 (LFA-1), were also comparable. Surface-antigen expression on marrow cells from the knockout mice was similar to that of wild-type mice, with the exception that most B220⁺ cells in knockout marrow were Mac-1⁺, a finding of uncertain functional relevance. We conclude that CD43 is not essential for haematopoiesis.

Previous studies have shown that monoclonal antibodies to human CD43 can be co-stimulatory for T cells¹² or activate them directly²⁰. More recently, expression of human CD43 by a murine T-cell hybridoma was observed to enhance its response to antigen¹⁵. We therefore tested T cells from the CD43-knockout mice and their wild-type littermates for their ability to proliferate in response to T-cell activators. Splenocytes were stimulated with the T-cell mitogen concanavalin A (conA), the anti-CD3 monoclonal antibody 145-2C11, or the superantigen staphylococcal enterotoxin B (SEB) at various concentrations. Thymocytes were stimulated with anti-CD3 and phorbol-12-myristyl-13-acetate (PMA). In each case, T cells from knockout mice showed substantially enhanced proliferation compared with those from their wild-type littermates (Fig. 2a). Moreover, enhanced proliferation was evident at all concentrations of stimulants tested, although maximal enhancement was seen with suboptimal concentrations of stimulants (not shown). To assess the dynamics of proliferation of CD43⁺ and CD43[−] T cells, different cell numbers were tested for proliferation at different time points using various concentrations of conA. At 48 h, the enhancement in proliferation of the CD43-deficient T cells was most striking when either conA concentration or cell number was limiting (Fig. 2b). By contrast, under optimal stimulatory conditions (2×10^5 cells, $2 \mu\text{g ml}^{-1}$ conA), only a modest increase (50%) in ³H-thymidine incorporation by the CD43-deficient cells was observed. However, at 24 h, duplicate cultures under identical stimulatory conditions showed a 300% increase in ³H-thymidine incorporation by the CD43-deficient cells (not shown). These results suggest an accelerated kinetics of T-cell activation in the absence of CD43.

To determine whether the proliferation of CD43-deficient T cells was also enhanced in response to a natural antigen, alloreactivity of T cells was tested. Again, proliferation of T cells from the CD43-knockout mice was more than double that of cells from their wild-type counterparts ($23.1 \pm 1.8 \times 10^3$ c.p.m. compared with $9.4 \pm 0.7 \times 10^3$ c.p.m., respectively). A similar increase in CD43-deficient T-cell proliferation was also seen after secondary and tertiary stimulation (not shown), indicating an increase in the proliferative ability of the CD43-deficient T cells rather than in the number of lymphocytes in the allospecific precursor pool.

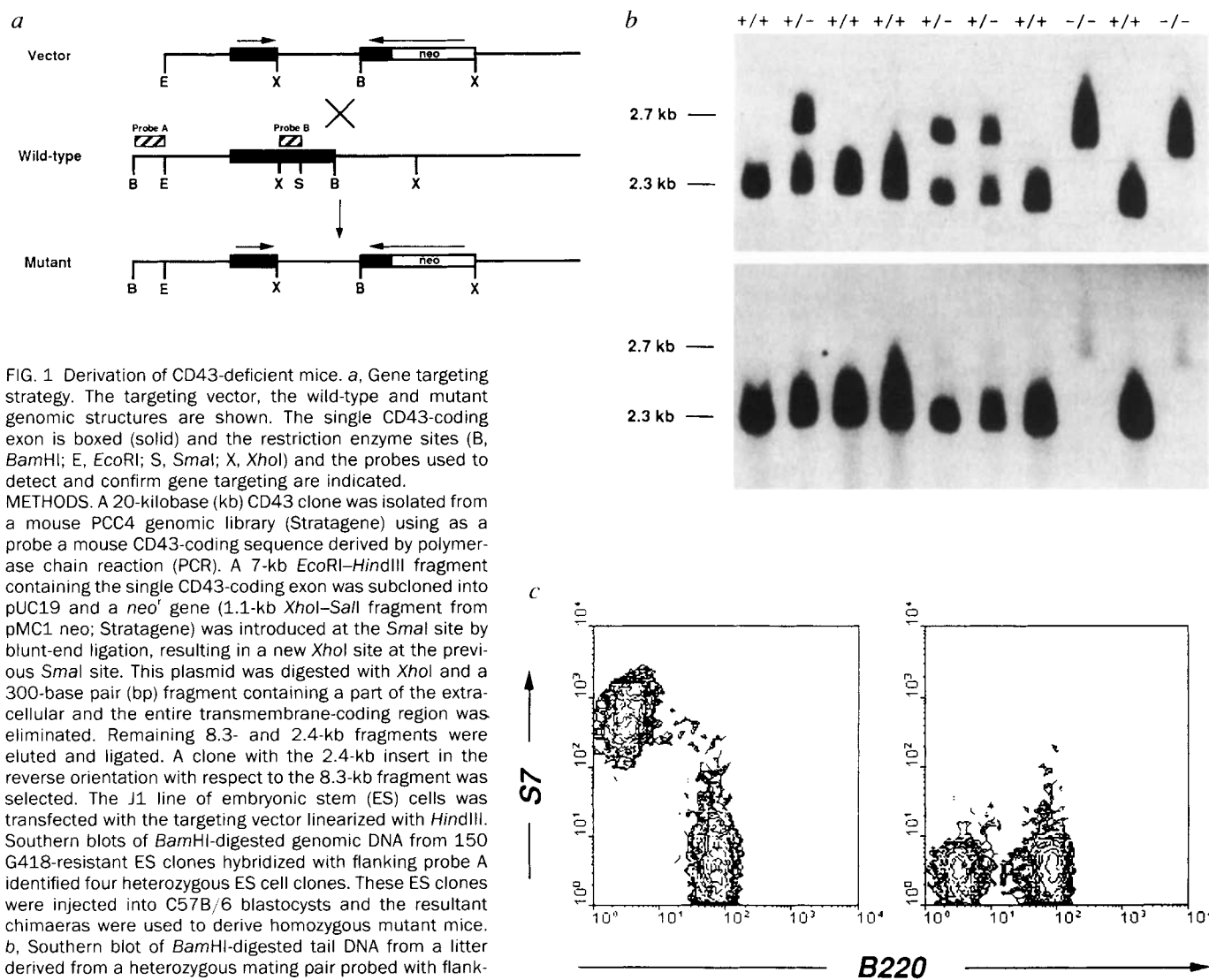
To investigate possible *in vivo* consequences of CD43-deficiency, cytotoxic T-cell response to vaccinia virus infection in the knockout mice was compared with that in their wild-type littermates. Seven days after infection with a high virus dose (10^6 TCID₅₀ (half-maximal tissue culture infective dose)), comparable levels of anti-vaccinia cytotoxic T-lymphocyte (CTL) activity were found in both mouse groups (not shown). By contrast, when limiting amounts of virus (2×10^3 TCID₅₀) were used, a significantly greater (fourfold) CTL response was consistently observed in the CD43-deficient mice (Fig. 3a). These

results are consistent with a decreased threshold for T-cell activation during an immune response. To determine the effect of the enhanced CTL activity in viral clearance, lungs and gonads from the mice were analysed for viral content. Surprisingly, infectious virus was recovered from all four CD43-knockout mice but from none of their wild-type littermates (Fig. 3b). These results suggest that defects involving tissue localization of effector cells or post-lysis viral clearance by phagocytes might occur in the absence of CD43.

The intercellular adhesion molecule ICAM-1 is reported to be a ligand for CD43 (ref. 14), suggesting that CD43 may mediate intercellular adhesion. In other studies, however, leukocytes from CD18-deficient (LAD) individuals showed no binding to ICAM-1, despite surface CD43 expression²¹, and the anti-CD43 monoclonal antibody previously observed to block CD43 binding to ICAM-1 did not block binding of CD43⁺ lymphoid cells to ICAM-1 or ICAM-3²². We have observed that CD43 expression impedes homotypic and heterotypic adhesion of cells, and diminishes cell binding to purified ligands^{23,24}. As interaction of T lymphocytes with other cells (for example, antigen-presenting cells) is required for optimal activation^{25,26}, the enhanced sensitivity of the CD43-deficient T lymphocytes to

activation might reflect increased adhesive properties. Thus, cells from the CD43-knockout mice and wild-type littermates were compared in homotypic adhesion assays and for their ability to bind purified ligands, including murine fibrinogen and soluble, recombinant ICAM-1²⁷. PMA-treated thymocytes from knockout mice showed substantially increased homotypic adhesion compared with thymocytes from wild-type littermates (Fig. 4a). Furthermore, binding of CD43-deficient splenic T cells was nearly 100% greater than binding of CD43⁺ cells to fibronectin (Fig. 4b), and 50% greater to ICAM-1 (Fig. 4c). The binding of CD43⁺ and CD43⁻ cells to ICAM-1 was mediated through LFA-1 because in both cases it was completely blocked by the anti-LFA-1 antibody M17/4. Taken together, these data indicate that loss of CD43 expression by T lymphocytes results in hyperadhesive properties and suggest that the increased sensitivity of CD43-deficient T cells to activation is related to their enhanced adhesiveness.

It has been suggested that structural features of CD43 are consistent with an anti-adhesive function^{3,28}. The mucin-like extracellular CD43 domain probably exists as an unfolded structure³, and has been measured to extend 45 nm in length²⁹, a distance greater than spanned by most cell-surface adhesion



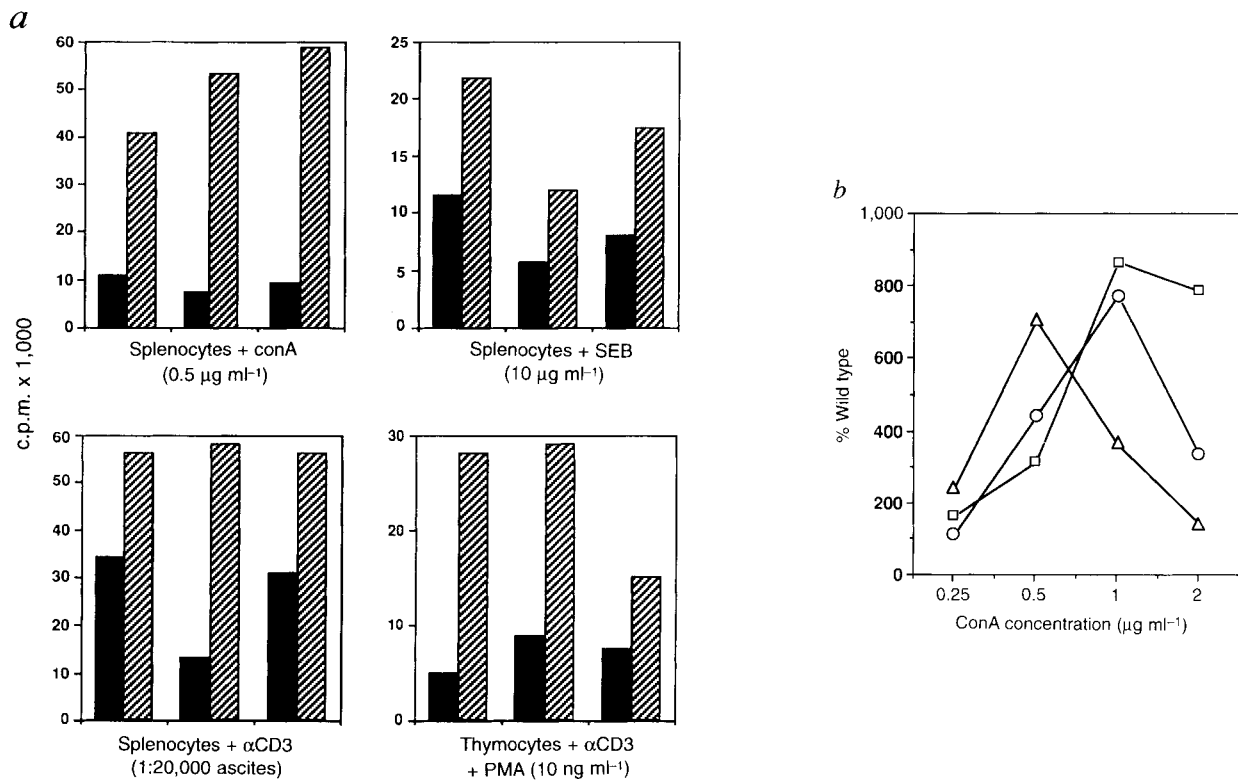


FIG. 2 Proliferative response of T lymphocytes from CD43-knockout mice. *a*, Data from three pairs (representative of six pairs tested) of wild-type (+/+; ■) littermates and CD43-knockout (-/-; ▨) mice are shown. 2×10^5 splenocytes per well were stimulated in 96-well culture plates with the indicated concentrations of conA, SEB or the anti-CD3 ascites 145-2C11. Thymocytes (2×10^5 per well) were stimulated with 145-2C11 and PMA. The cultures were pulsed with ^3H -thymidine for 42–48 h (0.5 μCi per well) and cells were collected onto 96-well GF/C

plates and counted in a Packard microplate scintillation counter. Results shown are after subtraction of ^3H -thymidine incorporation in nonstimulated cultures. All assays were done in triplicate. *b*, Dynamics of proliferative response. Varying numbers of splenocytes from +/+ and -/- mice were stimulated with different concentrations of conA as indicated and proliferation was measured as described above. Data are shown as per cent counts obtained with +/+ splenocytes. Δ , 2.0×10^5 splenocytes; $\circ$, 1.0×10^5 ; $\square$, 0.5×10^5 .

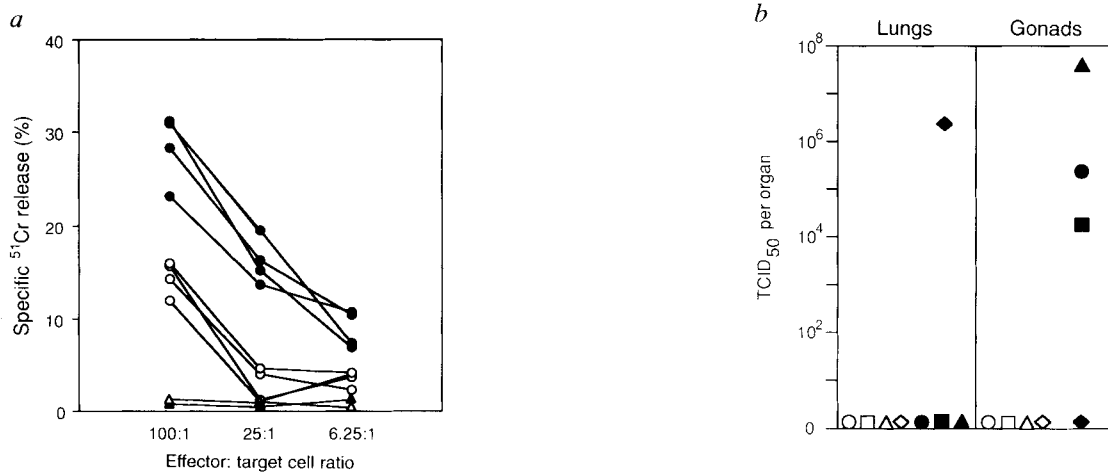


FIG. 3 CTL response to vaccinia virus infection. *a*, Mice were infected by intraperitoneal injection with 2×10^5 TCID₅₀ of vaccinia virus (WR strain). On day 7 after infection, splenocytes were depleted of B cells with B220-coated magnetic beads and assayed for specific lysis of MC57G target cells uninfected (triangles) or infected (circles) with vaccinia virus (10 TCID₅₀ per cell) for 3 h before labelling with ^{51}Cr . The

percentage specific release of ^{51}Cr was calculated from the (experimental release - spontaneous release) $\times 100$ / (total release - spontaneous release). *b*, Vaccinia virus titre. TCID₅₀ in lungs and gonads obtained from mice killed for CTL assay was measured on CV1 indicator cells. Different symbols represent individual mice (solid symbols indicate -/- mice, open symbols indicate +/+ mice).

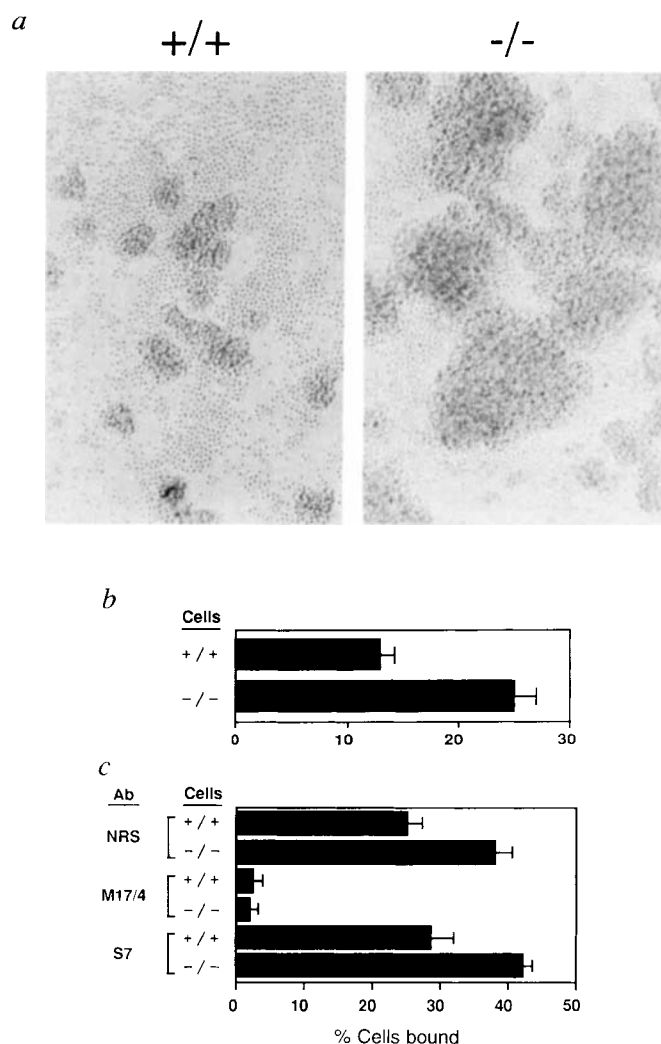


FIG. 4 a, PMA-induced homotypic adhesion of thymocytes; b, Binding of splenic T cells to fibronectin, and c, to ICAM-1.

METHODS. For homotypic adhesion assays, 2×10^6 thymocytes from +/+ and -/- mice in 1 ml of medium (RPMI 1640 with 10% FBS) were added to individual wells in a 24-well plate. PMA (40 ng ml⁻¹ final concentration) was added to each well and cells were photographed (200 $\times$ magnification) after 1 h incubation in a humidified incubator at 37 °C, 5% CO₂. For adhesion to purified ligands, splenocytes from +/+ and -/- mice were depleted of B cells and macrophages using anti-B220 monoclonal antibody (clone RAB-632; Pharmingen) coupled to immunomagnetic beads followed by plastic adherence for 2 h at 37 °C. Non-bead-bound, non-adherent cells were then treated with anti-CD3 ascites (mAb 145-2C11 at 1:1,000 dilution) for 30 min at 4 °C. Cells were labelled with carboxyfluorescein dye (BCECF; Molecular Probes), washed in adhesion buffer (RPMI, 5% FBS) and resuspended at 10^6 per ml in adhesion buffer containing PMA at 40 ng ml⁻¹. Polystyrene plates (96-well flat-bottom; Linbro) were prepared by coating overnight at 4 °C with murine fibronectin or sICAM-1 (ref. 25), both at 4 μ g ml⁻¹ in 0.02 M Tris-buffered saline (TBS), pH 7.2, or with buffer alone. The binding assay was carried out as described²⁴. For antibody inhibition studies, cells were incubated with a rat anti-mouse LFA-1 (clone M17/4; Pharmingen), rat anti-mouse CD43 (clone S7) at 20 μ g ml⁻¹, or normal rat serum (1:500) at room temperature for 10 min before adding to ligand-coated wells. The percentage of bound cells was calculated from (fluorescence of cells in ligand-coated wells - fluorescence of cells in buffer-coated wells) $\times$ 100/(fluorescence of cells added to the wells). All tests were performed in triplicate. Data shown are from one experiment and are representative of three separate assays.

molecules and their respective counter-receptors²⁸. Moreover, the substantial number of sialic acid residues in the CD43 extracellular domain^{3,5,30} probably gives the molecule a gross negative charge. These features might explain the ability of CD43 to retard T-lymphocyte interactions, which in turn might impede

cell activation. It is also possible, however, that CD43 acts more directly to suppress T-cell activation. Structure-function analysis using cytoplasmic domain truncations should elucidate whether the anti-adhesive and -proliferative phenotypes are related and clarify the mechanism of CD43-regulated T-cell activation. □

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Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice

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THE intracellular protein tyrosine kinase FAK (focal adhesion kinase) was originally identified by its high level of tyrosine phosphorylation in *v-src*-transformed cells¹⁻⁴. FAK is also highly phosphorylated during early development^{5,6}. In cultured cells it is localized to focal adhesion contacts and becomes phosphorylated and activated in response to integrin-mediated binding of cells to the extracellular matrix, suggesting an important role in cell adhesion and/or migration. We have generated FAK-deficient mice by gene targeting to examine the role of FAK during development. Mutant embryos displayed a general defect of mesoderm development, and cells from these embryos had reduced motility *in vitro*. Surprisingly, the number of focal adhesions was increased in FAK-deficient cells, suggesting that FAK may be involved in the turnover of focal adhesion contacts during cell migration.

The FAK gene was mutated by inserting the neomycin phosphotransferase gene (*neo^r*) in the beginning of its kinase domain⁷ (Fig. 1a). Heterozygous mice were normally obtained, and abnormal embryos were found around the eighth day *post coitum* (E8.0) when they were crossed with each other. Genotype analyses proved all of these to be homozygous mutants (Fig. 1b), and the FAK protein, of relative molecular mass (*M_r*) 125K, was absent in them (Fig. 1c). In addition, neither carboxy-terminal truncated products nor the induction of FAK-related non-kinase (FRNK)⁸ expression were detected. Furthermore, transcripts from the amino-terminal region were not detected by reverse transcription-polymerase chain reaction (RT-PCR) analyses⁷, nor were any translated products from that region found with an antibody against the N-terminal part of the FAK protein (Fig. 1c). Thus the *neo^r* insertion appeared to have caused the null mutation. FAK expression was ubiquitous in normal embryos at this stage, but was more intense in mesoderm⁹.

The E7.5 homozygous mutant embryos were reasonably normal: in the absence of FAK, embryos were able to implant and initiate gastrulation. Abnormality was evident in E8.0 mutant embryos, however, and pronounced in E8.5 mutant embryos (Fig. 1d). Development of the anteroposterior axis was dramatically retarded, and that of mesoderm was poor: head mesenchyme was involuted, and no notochord or somite was formed. The detailed analysis of the mutant phenotype will be reported elsewhere⁹, but the abnormalities were characterized

as the general mesodermal defects. Head mesenchyme, lateral mesoderm, extra-embryonic mesoderm, heart and vasculature all initially developed normally, but growth was subsequently retarded. There was also a variation in phenotype: some embryos did not develop headfold at all. The overall phenotype was strikingly similar to the fibronectin-deficient phenotype¹⁰, suggesting that FAK uniquely mediates fibronectin-integrin interactions in cellular processes at this stage of development.

The mesodermal defects might be due to impaired cell growth. To examine this possibility, mesodermal cells were isolated from the E8.0 embryos¹¹, and their dividing potential was determined by time-lapse photography. There was no difference in the number of cells divided at 10 h: 23 of 83 (28%) normal cells, and 27 of 99 (27%) mutant cells divided. FAK deficiency was also unlikely to impair differentiating potency of mesodermal cells *per se*. The mutant embryos expressed the *Brachyury* (T) gene⁹, and a few mutant embryos that were most advanced had a well-organized notochord. The mutant embryos expressed *mox-1* (ref. 9), a marker for somitogenetic mesoderm, and a few pairs of somitic condensations were histologically identified in some embryos, although they were hypomorphic and showed no sign of de-epithelialization. Thus somitogenetic potency and the ability to form notochord appeared to be retained in FAK-deficient mesoderm. In addition, as reported previously⁷, a variety of mesodermal cells differentiated normally from FAK-deficient (double-knockout) embryonic stem cells *in vitro* by retinoic acid and embryoid body formation, and *in vivo* under the skin of nude mice.

To elucidate the cellular basis of abnormalities in FAK-deficient embryos, fibroblast-like cells were cultured from E8.0 embryos (Fig. 2 legend). FAK(-/-) cells were more rounded and poorly spread than FAK(+/+) cells (Fig. 2c). Unexpectedly, attachment of FAK(-/-) cells to fibronectin was only slightly impaired. FAK(+/+) cells did not attach to laminin or collagen I, but FAK(-/-) cells did attach to these substrates, albeit inefficiently. Both FAK(+/+) and FAK(-/-) cells attached to vitronectin (data not shown). Mobility of the cells was diminished in FAK(-/-) cells, however, when examined by Boyden chamber assay with fibronectin as a substratum¹²; this was apparent in short-term, but not long-term, culture (Fig. 2a). To confirm this, mesodermal cells were isolated from E7.5 wild-type and apparently normal FAK-deficient embryos, and mobility was measured directly by time-lapse photography¹¹ (Fig. 2b). Wild-type cells exhibited a typical polar migratory shape (Fig. 2c) and moved around actively at an average speed of 83 $\mu\text{m h}^{-1}$. Mutant cells did not display a polar migratory conformation, retaining a more rounded cell shape (Fig. 2c), and had dramatically reduced mobility, with an average speed of 42 $\mu\text{m h}^{-1}$. Thus it appears that FAK-deficient embryos experienced deterioration because of failure of the organization of tissue architecture, resulting from a defect in mesodermal migration. However, mobility of the cells was not completely abolished even in the absence of FAK; this residual mobility might explain the variation in phenotype.

To investigate the mechanism leading to impaired migratory activity by FAK deficiency, we examined the organization of the cytoskeleton. Rhodamine-phalloidin staining of the fibroblast-like cells plated onto fibronectin-coated dishes indicated that there were stress fibres in the FAK(-/-) cells (Fig. 3b, c). However, actin fibres were dense all around the cell periphery rather than just in the central part, as compared with those in the FAK(+/+) cells (Fig. 3a). In addition, numerous microspikes were present all around the FAK(-/-) cell perimeter. These are normally found in the early stage of non-polar cell spreading^{13,14}. FAK(-/-) cells also had more diffuse subcortical distribution of cortactin, an actin-binding protein¹⁵, indicating a disorganization of the cortical cytoskeleton (Fig. 3d, e).

The non-polar cell spreading in FAK(-/-) cells could be due to failure in the formation of focal adhesions. This was first examined by staining with antibodies against talin and vinculin,

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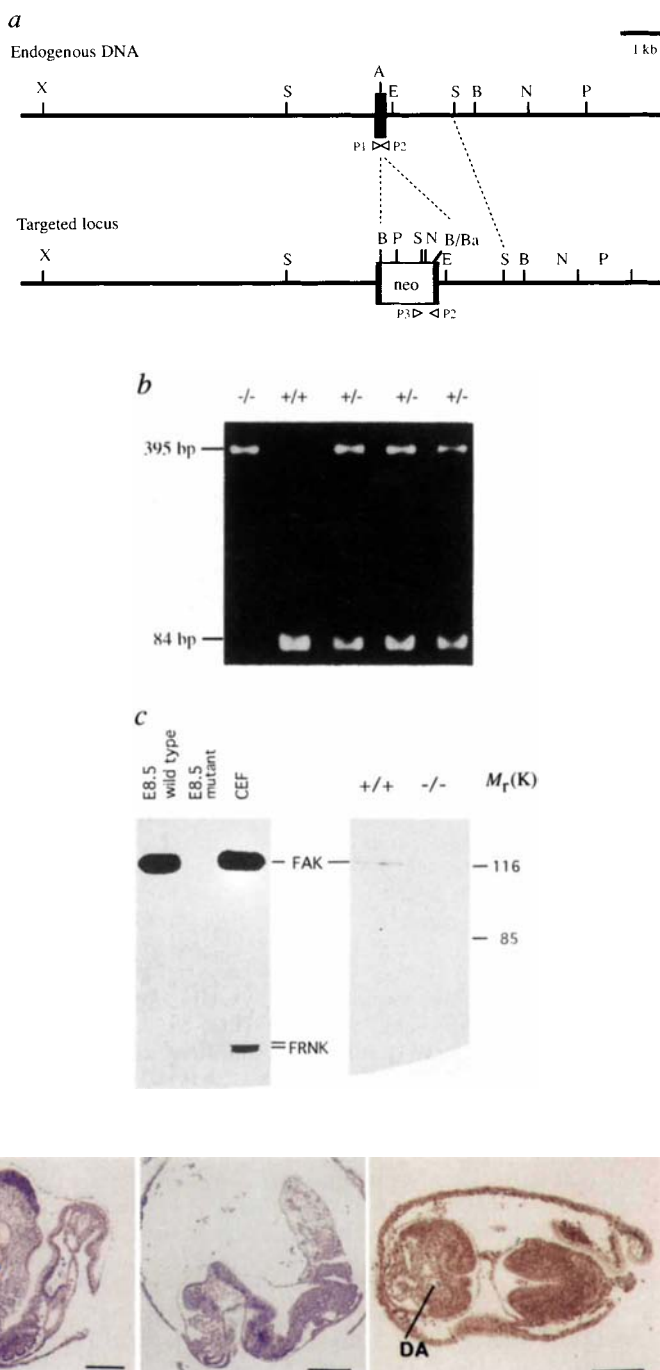
which are major components of focal adhesions. In FAK(-/-) cells they were stained as patches scattered all over the ventral surfaces facing the substrate (Fig. 3g, i). Under the same conditions in FAK(+/+) cells, talin- and vinculin-positive patches were found at the cell periphery in the form of typical focal adhesions (Fig. 3f, h). There were several times as many patches in FAK(-/-) cells than in FAK(+/+) cells. Proteins phosphorylated on tyrosine are also normally concentrated in focal adhesions⁶. Their distribution in FAK(-/-) cells with anti-phosphotyrosine antibodies (Fig. 3j, k) was similar to the distribution of talin and vinculin. FAK is thought to be involved in the formation of focal adhesions¹⁶, which raised the question of whether the patches in FAK(-/-) cells represent premature contacts or focal adhesions. Interference reflection microscopy (IRM) was used to analyse this¹⁷, together with immunofluo-

rescent staining for vinculin and actin filaments. In FAK(+/+) cells, stress fibres were terminated at the arrowhead-like vinculin-positive patches in ruffling borders and lamellipodia, and these patches were superimposed on black regions of the IRM image (data not shown), as reported previously^{17,18}. In FAK(-/-) cells, randomly oriented stress fibres were also terminated at numerous vinculin-positive patches throughout the ventral plasma membrane, as well as those in the cell periphery, where there were black regions of the IRM image (Fig. 3j-l), confirming the formation of focal adhesions in FAK(-/-) cells. Perhaps not only the formation of focal adhesions but also their turnover is essential for cell spreading and movement. The result indicates that FAK is involved in the turnover of focal adhesions, rather than in their formation, and that the cells were retained in the non-polar cell state by tighter adhesion

FIG. 1 Mesodermal defects by FAK deficiency. **a**, Schematic representation of normal and mutated FAK alleles. Black boxes represent the targeted exon spanning base pairs 1,442^{Thr}-1,525^{Thr} (ref. 3). P1, P2 and P3 indicate locations of primers used in the genotyping of embryos and mice by PCR. Only relevant restriction enzyme sites are indicated: X, *Xho*I; S, *Sph*I; A, *Afl*III; B, *Bgl*III; Ba, *Bam*HI; N, *Nco*I; P, *Pst*I; E, *Eco*RI.

b, An example of PCR identification of wild-type (+/+), heterozygous (+/-) and homozygous (-/-) mutant embryos. **c**, Western blot analyses of FAK expression in E8.5 normal and mutant embryos (left) with the antibody JF1 raised against the C-terminal part of mouse FAK (GST-FAK fusion protein, where FAK corresponds to 2,375-3,670 bp of murine complementary DNA³), and (right) with an antibody against the N-terminal part of FAK protein (UBI). JF1 detected FRNK in chick embryonic fibroblasts (CEF), but it was absent in normal and mutant mouse embryos; FRNK is a 41-43K protein which represents the C-terminal non-kinase region of FAK⁸. The similar results were obtained with the antibody BC3 against the C-terminal part (amino acids 392-406; UBI)⁸. **d**, Histological appearance of E8.5 mutant embryos. A parasagittal view of normal embryo (left), a sagittal view of an advanced mutant embryo (middle), and a transverse view of an advanced mutant (right). Headfold was deformed with poor head mesenchyme, and no notochord (Nc) or somites were formed. Cardiac plates fused at the centre to form the heart bulge, but the differentiation of meso- and endocardium was not apparent. Dorsal aorta (DA) was present in the anterior region, but was obscured in the caudal region. Omphalomesenteric artery was not apparent at the caudal extremity of the embryo. The neuroectoderm had multiple bends and distortions, but the neuroepithelium was reasonably thick and had many active mitotic figures. Deformity of neuroectoderm appeared to be a secondary defect resulting from insufficient support by poorly developed mesoderm.

METHODS. Three homologous recombinant ES clones were isolated with TT2 ES cells^{7,26}, and no difference was found in phenotype among mouse strains independently established from them²⁶. The heterozygotes were crossed with each other, DNAs were isolated from the yolk sac²⁷, and the genotype of embryos was assessed by PCR analysis using primers P1 (5'-GAGAATCCAGCTTTGGCTGT-3') and P2 (5'-GGCTCTTGAAGGAACCTCTC-3') to detect an 84-bp band for the wild-type allele, and primers P2 and P3 (5'-CAGGATGATCTGGACGAAGA-3') to detect a 395-bp band for the recombinant allele. For western blot analyses, mouse embryos were made soluble in TNE buffer²⁷. Proteins (20 µg per lane) were subjected to SDS-PAGE and probed with anti-FAK antibodies JF1, BC3 (ref. 8) or with antibody raised against the N-terminal 392-406 amino-acid sequence of murine FAK (UBI), all at a dilution of 1:2,000.



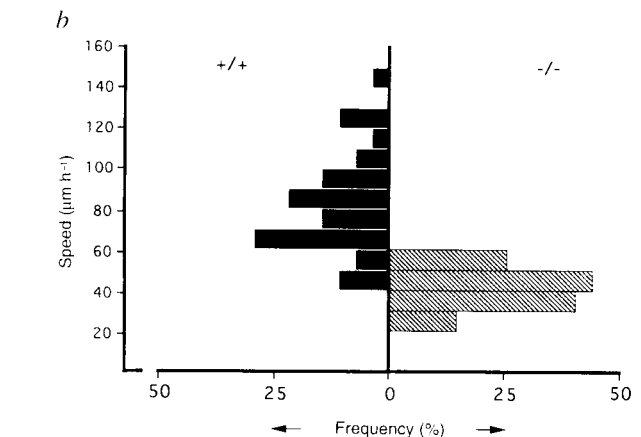
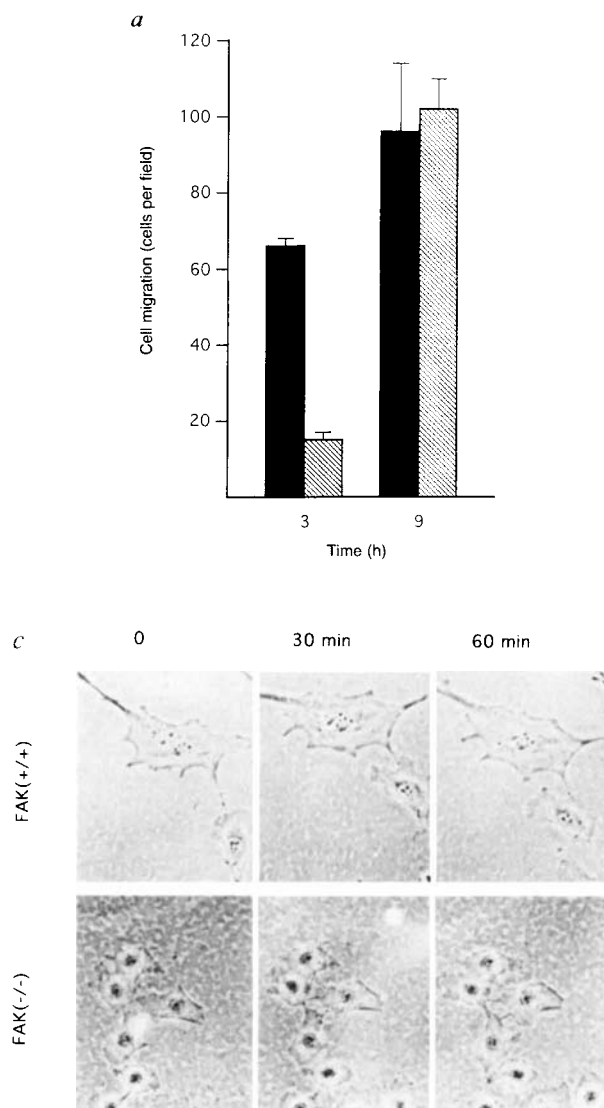


FIG. 2 Decrease in cell mobility. **a**, Migration of FAK(+/+) (black columns) and FAK(-/-) (shaded columns) cells on fibronectin (FN) by Boyden chamber assay. **b**, Time-lapse photography of migratory potentials of embryonic mesodermal cells on FN-coated dishes. Left (black) and right (cross-hatched) columns give the distribution of wild-type and mutant cells, respectively, that migrated at the indicated speeds. The average speed of migration was $83 \mu\text{m h}^{-1}$ for wild-type and $42 \mu\text{m h}^{-1}$ for mutant cells. **c**, Migratory shapes of mesodermal cells. Cells photographed at 30-min intervals are shown to illustrate the polar shape of cells from normal embryos and the rounded, non-polar shape of cells from FAK-deficient embryos.

METHODS. To obtain FAK(+/+) and FAK(-/-) cell cultures, a p53 mutation²⁸ was introduced into FAK-deficient embryos. The p53 deficiency enhances the proliferative capacity in a variety of types of cells *in vitro*²⁸, but p53 is unlikely to be involved directly in cellular events in which FAK functions. The cells were cultured in 10% FCS-DMEM (GIBCO) and analysed at early passages to minimize changes with culture. No difference was found in the rate of cell division between FAK(+/+) and FAK(-/-) cells. The Boyden chamber assay was performed as described². Isolation of the mesodermal cell layer from E7.5 embryos that did not have the p53 mutation, time-lapse video recording and analyses of migration distances were all performed as described previously¹¹. A single recording (1 s) was repeated at intervals of 1 min, and the rate of movement was determined by tracing the centre of the nucleus of each cell every 10 min for 10 h. In total, 32 and 33 cells from wild-type and mutant embryos, respectively, were examined.

to substrate. The opposite effects on focal adhesions of FAK phosphorylation by binding of the extracellular matrix to integrins⁶ and by *v-src*¹⁸ might now be considered as their rapid turnover by *v-src*, in contrast to moderate turnover by integrin stimulation.

Tyrosine phosphorylation of proteins was examined to investigate the molecular events underlying the increase in focal adhesion formation. The analyses indicated a higher level of tyrosine phosphorylation in several proteins as a result of FAK deficiency (Fig. 4a); it may reflect enhanced formation of focal adhesions and microspikes. For example, the 110K protein was not FAK-B (ref. 19), Src-substrate p120 (ref. 20) or Cbl (ref. 21) (data not shown), but appeared to be brush-border myosin I (BB myosin I), which is localized in microspikes²² (Fig. 4b). In contrast, no proteins were found in which tyrosine phosphorylation was decreased in FAK(-/-) cells and which would thus be substrates of FAK in normal cells. The components of focal adhesion are obviously candidates for FAK substrates.

We then examined the tyrosine phosphorylation status of vinculin, talin, paxillin, tensin and α -actinin (Fig. 4b). The expression of many of these appeared to be enhanced in FAK(-/-)

cells, as they coincided with increases in the number of focal adhesions. No change in the level of phosphorylation was detected, however. Cortactin, which was disorganized in the subcortical region of FAK(-/-) cells (Fig. 3d, e), also showed no change in the tyrosine phosphorylation level (Fig. 4b). However, a 90K molecule co-immunoprecipitated with α -actinin appeared to have a remarkably reduced tyrosine phosphorylation in FAK(-/-) cells (Fig. 4b).

The β_1 and β_3 integrins have been suggested to associate directly with FAK, and the association might be affected by phosphorylation of integrins and FAK^{16,31}. No changes in the expression of β_1 , β_3 or several α -subunits (α_2 , α_3 or α_4) that form complexes with β_1 were found by FAK deficiency (Fig. 4c). There was no tyrosine phosphorylation of these integrin β -subunits in either FAK(+/+) or FAK(-/-) cells, and no significant change in the β_1 distribution was found by FAK deficiency (Fig. 3o, p).

Many questions remain to be answered about the molecular events caused by FAK deficiency. The direct substrates of FAK might be among other focal adhesion components and integrin subunits not examined in this study or not yet identified. Some non-receptor protein kinases have been reported to localize in

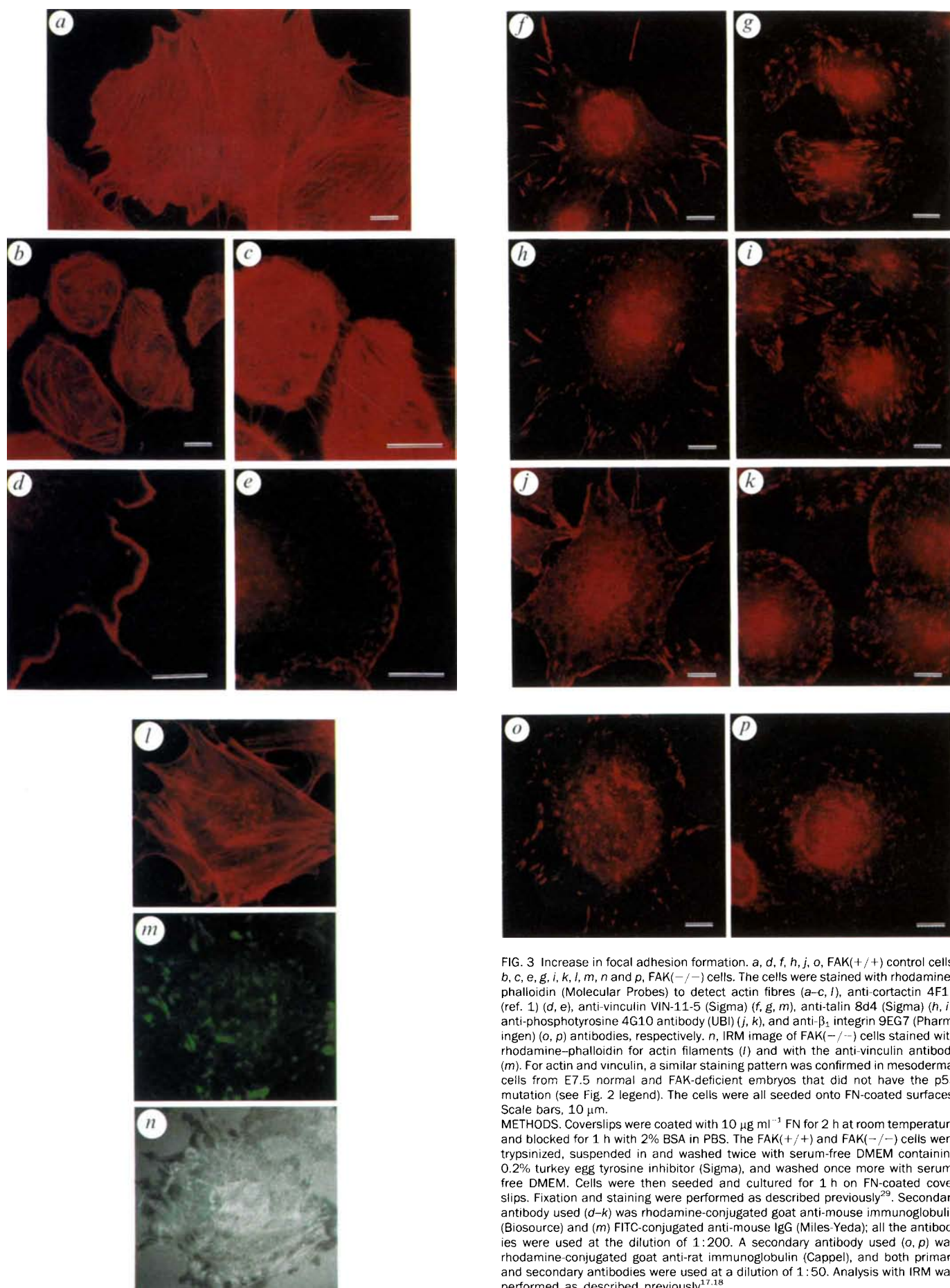


FIG. 3 Increase in focal adhesion formation. *a, d, f, h, j, o*, FAK(+/+) control cells; *b, c, e, g, i, k, l, m, n* and *p*, FAK(-/-) cells. The cells were stained with rhodamine-phalloidin (Molecular Probes) to detect actin fibres (*a-c, l*), anti-cortactin 4F11 (ref. 1) (*d, e*), anti-vinculin VIN-11-5 (Sigma) (*f, g, m*), anti-talin 8d4 (Sigma) (*h, i*), anti-phosphotyrosine 4G10 antibody (UBI) (*j, k*), and anti- β_1 integrin 9EG7 (Pharmingen) (*o, p*) antibodies, respectively. *n*, IRM image of FAK(-/-) cells stained with rhodamine-phalloidin for actin filaments (*l*) and with the anti-vinculin antibody (*m*). For actin and vinculin, a similar staining pattern was confirmed in mesodermal cells from E7.5 normal and FAK-deficient embryos that did not have the p53 mutation (see Fig. 2 legend). The cells were all seeded onto FN-coated surfaces. Scale bars, 10 μ m.

METHODS. Coverslips were coated with 10 μ g ml⁻¹ FN for 2 h at room temperature and blocked for 1 h with 2% BSA in PBS. The FAK(+/+) and FAK(-/-) cells were trypsinized, suspended in and washed twice with serum-free DMEM containing 0.2% turkey egg tyrosine inhibitor (Sigma), and washed once more with serum-free DMEM. Cells were then seeded and cultured for 1 h on FN-coated cover slips. Fixation and staining were performed as described previously²⁹. Secondary antibody used (*d-k*) was rhodamine-conjugated goat anti-mouse immunoglobulin (Biosource) and (*m*) FITC-conjugated anti-mouse IgG (Miles-Yeda); all the antibodies were used at the dilution of 1:200. A secondary antibody used (*o, p*) was rhodamine-conjugated goat anti-rat immunoglobulin (Cappel), and both primary and secondary antibodies were used at a dilution of 1:50. Analysis with IRM was performed as described previously^{17,18}.

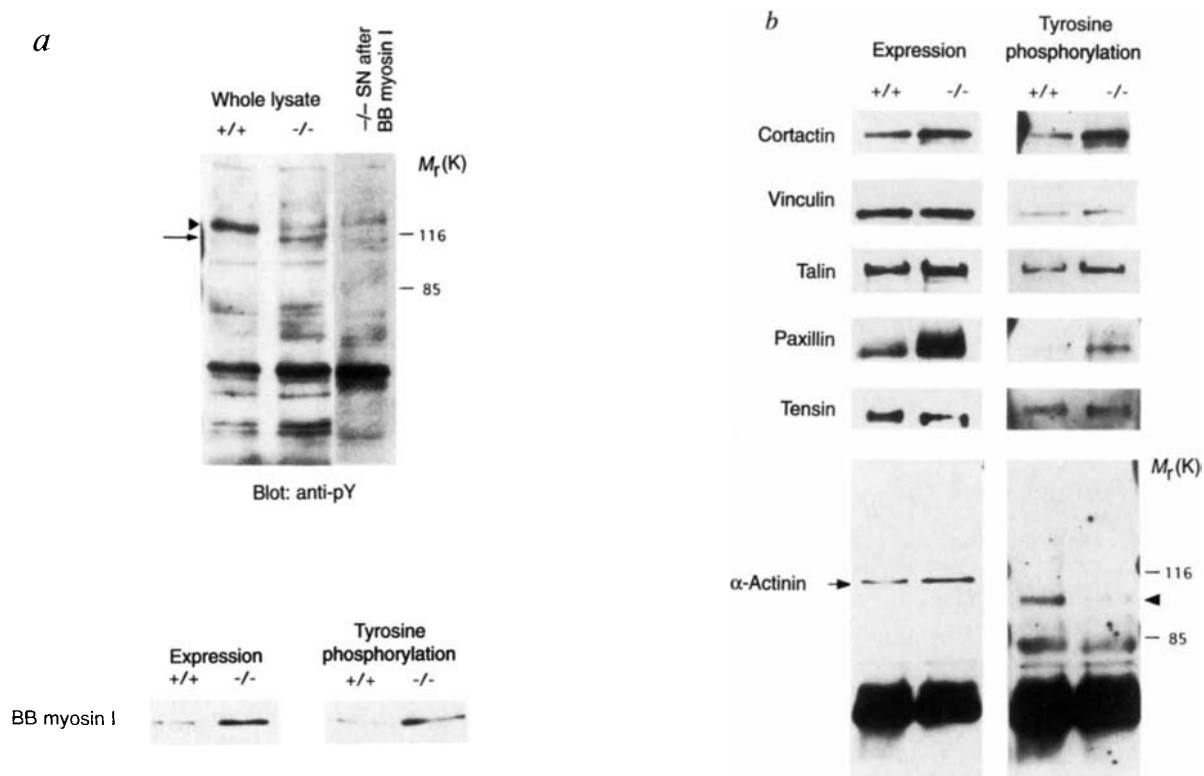


FIG. 4 Protein tyrosine phosphorylation in FAK(+/-) and FAK(-/-) cells. **a**, Analysis of tyrosine phosphorylation on whole-cell lysates (top, left and middle lanes) with anti-phosphotyrosine 4G10 antibody. The 125K phosphorylated band in FAK(+/-) cells that was lost in FAK(-/-) cells represents the FAK protein itself. The 110K–120K protein in which tyrosine phosphorylation was enhanced in FAK(-/-) cells appeared to be BB myosin I: the level of 110K BB myosin I expression was enhanced in FAK(-/-) cells (bottom, left), and consequently the total amount of tyrosine phosphorylation was increased (bottom, right), although the phosphorylation level itself seemed to be unaltered. Tyrosine phosphorylation in 110K protein was indeed diminished by immuno-depletion of BB myosin I from FAK(-/-) cell lysates (top, right). The proteins of M_r ~60K–80K, for which tyrosine phosphorylation was also enhanced have still to be identified. However, components of focal adhesions are highly tyrosine phosphorylated⁶, and FAK(-/-) cells appeared to have an increased number of focal adhesions (see **b**). **b**, Expression (left) and tyrosine phosphorylation (right) of cortactin, vinculin, talin, paxillin, tensin and α -actinin, respectively. **c**, Expression of β_1 , β_3 , α_2 , α_3 and α_4 integrin subunits. Tyrosine phosphorylation of β_1 or β_3 subunits was not detectable in either FAK(+/-) or FAK(-/-) cells. Arrowheads indicate FAK (**a**) and an unknown protein co-immunoprecipitated with α -actinin (**b**).

METHODS. Cells were cultured for 1 h on FN-coated dishes as described in Fig. 3 legend, washed with cold 1 mM Na_3VO_4 in TBS, and lysed with TNE buffer²⁷. Equal amounts of proteins were used in each experiment. They were immunoprecipitated from the cell lysates with relevant anti-

bodies and Sepharose-G (Pharmacia), run on 6% or 8% SDS–polyacrylamide gels and transferred onto nitrocellulose membranes (Schleicher & Schuell). The membranes were blotted with anti-phosphotyrosine 4G10 (UBI), anti-BB myosin I (Chemicon), anti-cortactin 4F11 (ref. 1), anti-vinculin VIN-11-5 (Sigma), anti-talin 8d4 (Sigma), anti-paxillin Z035 (Zymed), anti- α -actinin BM-75.2 (Sigma), anti- β_1 integrin 9EG7 (Pharmingen), and anti- β_3 , α_2 , α_3 and α_4 integrin antibodies. Tensin was analysed as described previously³⁰.

focal adhesions²³, but their status in a condition of FAK deficiency is also unknown. The Ras/GRB2/MAP kinase pathway may also affect focal adhesion formation^{24,25}. The FAK(-/-) cells will be a valuable resource for these studies. □

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A role for phosphatidylinositol transfer protein in secretory vesicle formation

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VESICULAR traffic in eukaryotic cells is characterized by two steps of membrane rearrangement: the formation of vesicles from donor membranes and their fusion with acceptor membranes. With respect to vesicle formation, several of the cytosolic proteins implicated in budding and fission have been identified. A feature common to all these proteins is that their targets, when known, are other proteins rather than lipids. Here we report, using a previously established cell-free system^{1,2} derived from a neuroendocrine cell line, the purification of cytosolic factors that stimulate the formation of constitutive secretory vesicles and immature secretory granules from the *trans*-Golgi network. One such factor, referred to as CAST1, was identified as the α and β isoforms of the mammalian phosphatidylinositol transfer protein (PtdIns-TP) (refs 3–5). The yeast PtdIns-TP, SEC14p (ref. 6), which has no sequence homology to mammalian PtdIns-TP (refs 7,8), was able to substitute for the mammalian PtdIns-TP in secretory vesicle formation. Our results suggest a highly conserved role for phosphoinositides in vesicle formation.

The efficiency of post *trans*-Golgi network (TGN) secretory vesicle formation in the cell-free system^{1,2} (see legend to Fig. 1) decreased when diluted, rather than undiluted (~ 4 mg ml⁻¹ protein), post-nuclear supernatant (PNS) was used (Fig. 1a). This was probably because of a reduction in cytosolic factors stimulating this process rather than the dilution of membranes, because in our cell-free system post-TGN secretory vesicle formation does not depend on vesicles fusing with the TGN (ref. 9) (A. Schmidt and W.B.H., unpublished observations). Indeed, supplementation of the diluted PNS with a protein fraction prepared from bovine adrenal medulla cytosol brought the efficiency of vesicle formation back to the value observed with undiluted PNS (Fig. 1b). This allowed us to fractionate the cytosol in order to search for factors that stimulated post-TGN secretory vesicle formation, that is, those that became limiting under the present experimental conditions.

Differential ammonium sulphate precipitation (Fig. 1c–e) revealed that stimulatory activity was largely recovered in the two fractions containing proteins precipitated by 45–60% and 60–75% saturated ammonium sulphate (Fig. 1c), but not in the fractions containing the vast majority of coatamer (30–45% saturation ammonium sulphate) (Fig. 1e). Stimulation of post-TGN secretory vesicle formation by the proteins in the 45–75% ammonium sulphate fraction was dependent on their concentration, approaching a plateau (corresponding to a $\sim$ fourfold stimulation) at 9 mg ml⁻¹ (data not shown). Chromatography of the proteins present in the 45–75% ammonium sulphate fraction on Q-Sepharose (Fig. 1f, g) revealed two peaks of stimulatory activity, referred to as CAST1 and CAST2 (for cytosolic activity stimulating TGN vesicle formation) (Fig. 1f).

Sequential chromatography using Phenyl Superose (Fig. 2a, b) and Superdex 200 (Fig. 2d, e) of the Q-Sepharose fractions containing CAST1 activity yielded a protein doublet of 34–35K (Fig. 2f) which copurified with CAST activity (Fig. 2d). Protein sequencing of tryptic fragments of the 34K polypeptide revealed its identity with the recently described β isoform of the mammalian PtdIns-TP (ref. 4) (Fig. 2j). On sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the 34K polypeptide purified from bovine adrenal medulla (Fig. 2i, lane 4) comigrated with the β isoform of the PtdIns-TP purified from bovine brain (Fig. 2i, lane 3), and the 35K polypeptide (Fig. 2i, lane 4) comigrated with the α isoform³ of PtdIns-TP (Fig. 2i, lane 2). In western blots, the 34K and 35K polypeptides were immunolabelled by an antibody recognizing both the α and β isoforms of PtdIns-TP (Fig. 2g, h). Immunoblotting of the fractions obtained after Phenyl Superose (Fig. 2a, c) and Superdex 200 (Fig. 2d, f–h) chromatography showed copurification of PtdIns-TP immunoreactivity and CAST activity. Together, these observations indicate that the 35K and 34K polypeptides correspond to the α and β isoforms of PtdIns-TP, respectively, and suggest that CAST1 is PtdIns-TP, a ubiquitous cytosolic protein³ able to associate with the Golgi complex¹⁰.

Further evidence for the identity of CAST1 and PtdIns-TP was obtained by the observation that the α and β isoforms of PtdIns-TP purified from bovine brain and the purified recombinant mouse PtdIns-TP α isoform were able to stimulate post-TGN secretory vesicle formation in the cell-free system using diluted PNS (Fig. 3). In this assay, the activities of the α and β isoforms from bovine brain were (1) indistinguishable from each other, (2) very similar to that of the recombinant mouse α isoform, and (3) in the same range as that of purified CAST1 (Fig. 2d) when compared on an equal protein basis (data not shown). The PtdIns-TP-induced increase in appearance of the sulphate-labelled marker proteins (hsPG and SgII) in the vesicle fraction² (see legend to Fig. 1) represented the stimulation of true post-TGN secretory vesicle formation rather than fragmentation of the TGN because tyrosylprotein sulphotransferase, a TGN-resident membrane protein¹¹, remained with the TGN membranes in both the absence and presence of added PtdIns-TP (data not shown).

The yeast PtdIns-TP is the product of the *SEC14* gene (SEC14p)⁶. Purified recombinant SEC14p also stimulated cell-free post-TGN secretory vesicle formation (Fig. 4a), although not to the same extent as mammalian PtdIns-TP (Fig. 4b). Both SEC14p and mammalian PtdIns-TP exhibit PtdIns/phosphatidylcholine transfer activity *in vitro*^{3,6}, and mammalian PtdIns-TP can partially correct SEC14p dysfunction in yeast¹². Mammalian PtdIns-TP and SEC14p do not share significant primary sequence homology^{7,8}. Hence, their stimulatory effect on post-TGN secretory vesicle formation is most probably related to their phospholipid transfer activity. Our data with SEC14p are fully consistent with previous considerations^{7,13,14} that the defect in constitutive secretion in the *sec14-1*^{ts} mutant might be at the level of secretory vesicle biogenesis, and support the general concept that the molecular machinery of membrane traffic is highly conserved from yeast to mammals.

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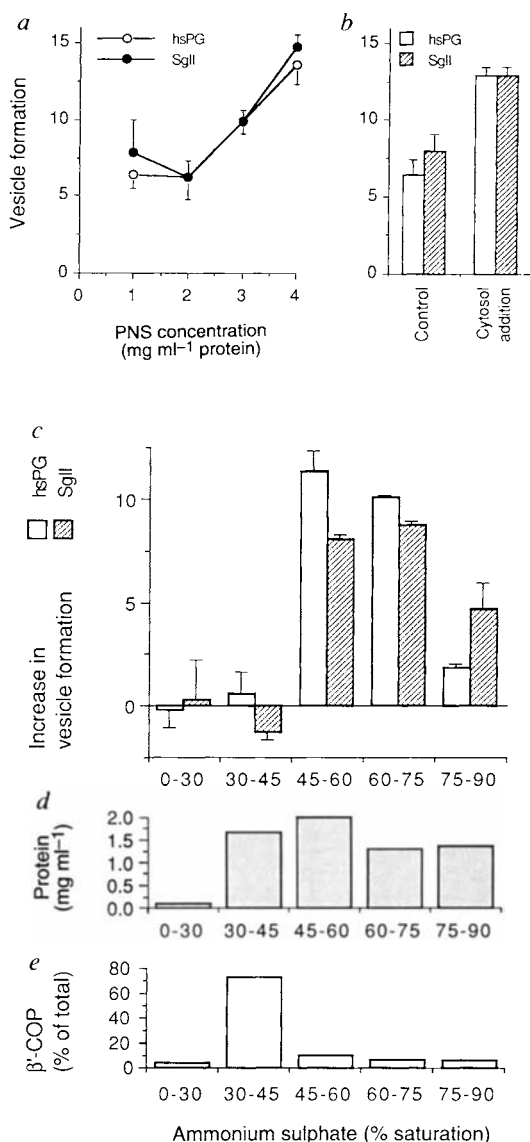


FIG. 1 Detection of two cytosolic activities (CAST) stimulating the formation of constitutive secretory vesicles and immature secretory granules (collectively referred to as post-TGN secretory vesicles) from the TGN in a cell-free system. **a**, Cell-free post-TGN secretory vesicle formation using post-nuclear supernatant (PNS) at various concentration. **b**, Cell-free post-TGN secretory vesicle formation using low concentration PNS (2 mg ml⁻¹ protein without (control) and with (cytosol addition) addition of a protein fraction (3 mg ml⁻¹) prepared from bovine adrenal medulla cytosol by precipitation with ammonium sulphate (90% saturation). **a** and **b**, Vesicle formation is expressed as described in methods and is given as mean $\pm$ s.e.m. ($n=3$). **c-e**, Fractionation of bovine adrenal medulla cytosol, using differential ammonium sulphate precipitation at the indicated percentages of saturation. **c**, CAST activity in the ammonium sulphate fractions. An equal proportion of each fraction was added to the PNS (2 mg ml⁻¹ protein) and assayed for CAST activity. Data are expressed as the increase in vesicle formation (see methods) above control reactions (PNS without addition) and are the mean of duplicate assays; bars indicate the variation of the individual values from the mean. **d**, Protein in the ammonium sulphate fractions. Data are expressed as the increase in protein concentration in the cell-free assay due to addition of the ammonium sulphate fraction. **e**, β' -COP immunoreactivity in the ammonium sulphate fractions. **f** and **g**, Q-Sepharose chromatography of an ammonium sulphate fraction (45–75% saturation) enriched in CAST (see **c**). **f**, An equal proportion of each Q-Sepharose fraction was added to the PNS (2 mg ml⁻¹ protein) and assayed for CAST activity. Data are expressed as the increase in vesicle formation (see methods) above control reactions (PNS without addition) and are the mean of duplicate assays; bars indicate the variation of the individual values from the mean. **g**, Protein in the Q-Sepharose

fractions. Data are expressed as the increase in protein concentration in the cell-free assay due to addition of the Q-Sepharose fractions. The dashed line indicates the KCl gradient used to elute the Q-Sepharose column.

METHODS. Cell-free post-TGN secretory vesicle formation using PC12 cell PNS, and separation of post-TGN vesicles from the TGN by differential centrifugation, were done as described previously², with the modifications described below. In this and the following figures, post-TGN secretory vesicle formation was determined by quantifying the transfer of marker proteins (heparan sulphate proteoglycan (hsPG) for constitutive secretory vesicles; secretogranin II (SgII) for secretory granules), sulphate-labelled in the TGN, into post-TGN secretory vesicles^{1,2}. Vesicle formation is defined as the amount of labelled marker protein in the P2 fraction² (post-TGN secretory vesicles) after incubation at 37 °C minus that at 4 °C, and is expressed as percentage of labelled marker in the P1 fraction² (TGN) kept at 4 °C. Either freshly prepared PNS or PNS that had been snap-frozen and stored at -80 °C were used, with similar results. The PNS (typical protein concentration ~4 mg ml⁻¹) was diluted with homogenization buffer plus sulphate (HBS) (ref. 1) to the indicated protein concentration, with or without addition of protein fractions prepared from bovine adrenal cytosol (see below). The assay volume was 50–130 μ l. All steps of protein fractionation of bovine adrenal medulla cytosol was performed at 4 °C. A 20,000g supernatant from bovine adrenal medulla was prepared as described previously¹¹, except that 1 mM phenylmethanesulphonyl fluoride was added to the homogenate. The supernatant was centrifuged at 200,000g for 90 min, the resulting supernatant adjusted to 4 mg ml⁻¹ protein, and protein precipitated by addition of solid ammonium sulphate to the indicated percentages of saturation. The precipitates were collected by centrifugation and resuspended in a minimum volume of either buffer A (10% (w/v) sucrose, 20 mM Tris-HCl, pH 7.4) (when subjected to chromatography) or HBS (when used in the cell-free assay). For the determination of CAST activity, the suspension was dialysed against HBS using Spectra/Por 2 membrane and insoluble material removed by ultracentrifugation. The cleared protein fractions were mixed with PNS (diluted to 2 mg ml⁻¹ protein) and the mixture subjected to cell-free post-TGN secretory vesicle formation. For further fractionation of the ammonium sulphate fraction (45–75% saturation) by Q-Sepharose chromatography, the precipitate resuspended in buffer A was dialysed against buffer A, cleared by ultracentrifugation, and 600 mg protein applied onto a 22 ml Q-Sepharose column (Pharmacia). Bound proteins were eluted with an 85 ml, 0–800 mM linear KCl gradient in buffer A at 1 ml min⁻¹, collecting 1 ml fractions. The fractions were combined as indicated in **f**, dialysed against HBS, and analysed for CAST activity as described above. Western blotting of the coatomer subunit β' -COP (ref. 26) was done using an antiserum according to standard methods using [¹²⁵I]protein A.

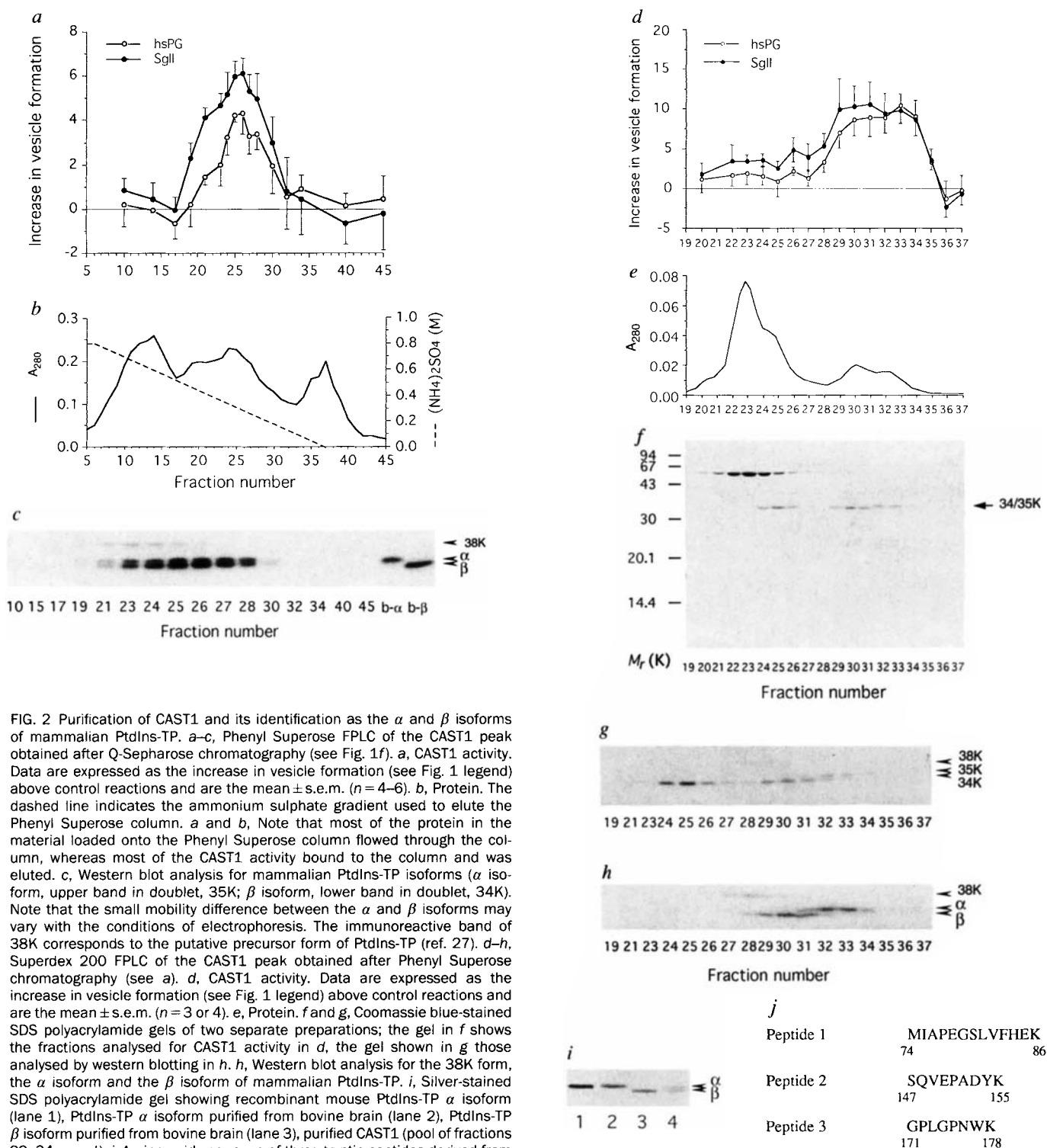


FIG. 2 Purification of CAST1 and its identification as the α and β isoforms of mammalian PtdIns-TP. **a–c**, Phenyl Superose FPLC of the CAST1 peak obtained after Q-Sepharose chromatography (see Fig. 1f). **a**, CAST1 activity. Data are expressed as the increase in vesicle formation (see Fig. 1 legend) above control reactions and are the mean $\pm$ s.e.m. ($n=4-6$). **b**, Protein. The dashed line indicates the ammonium sulphate gradient used to elute the Phenyl Superose column. **a** and **b**, Note that most of the protein in the material loaded onto the Phenyl Superose column flowed through the column, whereas most of the CAST1 activity bound to the column and was eluted. **c**, Western blot analysis for mammalian PtdIns-TP isoforms (α isoform, upper band in doublet, 35K; β isoform, lower band in doublet, 34K). Note that the small mobility difference between the α and β isoforms may vary with the conditions of electrophoresis. The immunoreactive band of 38K corresponds to the putative precursor form of PtdIns-TP (ref. 27). **d–h**, Superdex 200 FPLC of the CAST1 peak obtained after Phenyl Superose chromatography (see **a**). **d**, CAST1 activity. Data are expressed as the increase in vesicle formation (see Fig. 1 legend) above control reactions and are the mean $\pm$ s.e.m. ($n=3$ or 4). **e**, Protein. **f** and **g**, Coomassie blue-stained SDS polyacrylamide gels of two separate preparations; the gel in **f** shows the fractions analysed for CAST1 activity in **d**, the gel shown in **g** those analysed by western blotting in **h**. **h**, Western blot analysis for the 38K form, the α isoform and the β isoform of mammalian PtdIns-TP. **i**, Silver-stained SDS polyacrylamide gel showing recombinant mouse PtdIns-TP α isoform (lane 1), PtdIns-TP α isoform purified from bovine brain (lane 2), PtdIns-TP β isoform purified from bovine brain (lane 3), purified CAST1 (pool of fractions 29–34, see **d**). **j**, Amino-acid sequence of three tryptic peptides derived from the 34K polypeptide of CAST1.

METHODS. The CAST1 peak eluted from the Q-Sepharose column (see Fig. 1) was adjusted to 0.8 M ammonium sulphate, 30 mg of protein applied to a 1 ml Phenyl Superose column (Pharmacia), and bound protein eluted at 0.25 ml min⁻¹ with a 15 ml reverse ammonium sulphate gradient (from 0.8 M to 0 M) in 20 mM HEPES-KOH buffer (pH 7.2) containing 10% (w/v) sucrose. Fractions (0.5 ml) were collected, dialysed against HBS, and 15 μ l of each fraction were tested for CAST activity as described in the legend to Fig. 1, using a final assay volume of 65 μ l. For further Superdex 200 FPLC, Phenyl Superose chromatography was performed as described above except that 0.6 M rather than 0.8 M ammonium sulphate was used to increase the purity of the CAST1 peak eluting from the Phenyl Superose column. Fractions containing CAST1 activity were pooled, concentrated using Centricon 10, and 100 μ l (0.2 mg protein) applied to a Superdex HR 10/30 column (Pharmacia) equilibrated with HBS. FPLC was done at 0.25 ml min⁻¹ in HBS, and 0.4 ml

fractions were collected, starting with fraction 1, 12 min after injection of sample. Fractions (20 μ l) were tested for CAST1 activity as described in the legend to Fig. 1, using a final assay volume of 70 μ l. Fractions were subjected to SDS-PAGE using 12% gels (**c**, **g**, **h**, **i**) or 15% gels (**f**), and analysed by Coomassie Blue staining (**f**, **g**), silver staining (**i**), or western blotting (**c**, **h**) using an antibody against mammalian PtdIns-TP (ref. 10). **i**, The α and β isoforms of PtdIns-TP were purified from bovine brain (ref. 5 and K. J. V. *et al.*, manuscript in preparation). The recombinant mouse PtdIns-TP α isoform expressed in *Escherichia coli* was purified as described²⁸. **j**, The second half (later eluting) of the CAST1 peak obtained from the Phenyl Superose column was subjected to SDS-PAGE on a 15% gel, stained with Coomassie blue, and the gel piece containing the 34K band copurifying with CAST1 activity was excised from the gel and treated with trypsin. Tryptic digestion, extraction of the resulting peptides, HPLC-purification and sequence analysis were done as previously described²⁹.

FIG. 3 Purified α (b- α) and β (b- β) isoforms of bovine PtdIns-TP, and the purified α isoform of recombinant mouse PtdIns-TP (rm- α), stimulate cell-free post-TGN secretory vesicle formation. a, hsPG; b, SgII. Data are expressed as the increase in vesicle formation (see Fig. 1 legend) above control reactions and are the mean $\pm$ s.e.m. ($n = 5-8$).

METHODS. The α and β isoforms of bovine PtdIns-TP and the recombinant mouse PtdIns-TP α isoform were purified as described in the legend to Fig. 2, dialysed against HBS, and tested at the indicated concentrations for CAST activity as described in the legend to Fig. 1, using a final assay volume of 50 μ l.

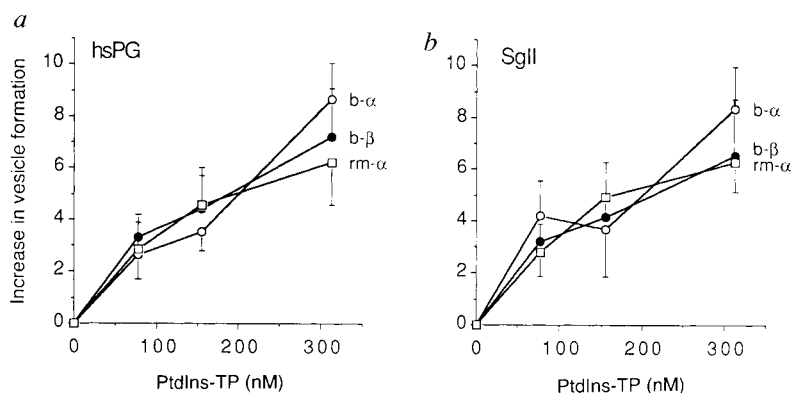
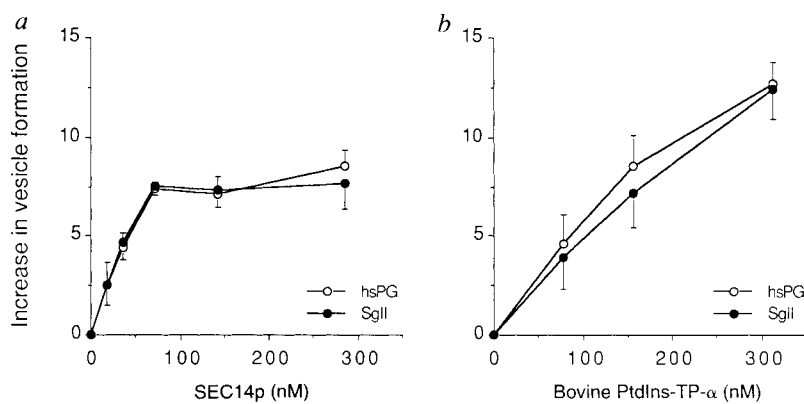


FIG. 4. Purified recombinant SEC14p stimulates cell-free post-TGN secretory vesicle formation (a). For direct comparison, the results of a parallel set of assays using the α isoform of PtdIns-TP purified from bovine strain are shown (b). Data are expressed as the increase in vesicle formation (see Fig. 1 legend) above control reactions and are the mean $\pm$ s.e.m. ($n = 6-9$).

METHODS. Recombinant SEC14p from *E. coli* was purified as previously described³⁰. Purified recombinant SEC14p and the purified α isoform of bovine PtdIns-TP were dialysed against HBS and tested at the indicated concentrations for CAST activity as described in the legend to Fig. 1, using a final assay volume of 50 μ l.



Martin and colleagues¹⁵ reported a role of mammalian PtdIns-TP in the ATP-dependent priming of secretory vesicles for fusion with the plasma membrane. Our observations reveal an additional role of mammalian PtdIns-TP in vesicular traffic: to promote formation of secretory vesicles from the TGN. The stimulation of vesicle formation by PtdIns-TP may well be related to its recently described¹⁶ role in polyphosphoinositide synthesis. Although we do not yet know the identity of CAST2, it is possible that this activity, given that post-TGN secretory vesicle formation requires ATP (ref. 1), and in analogy to secretory vesicle priming for fusion¹⁷, turns out to be an inositol lipid kinase. Transiently increased levels of polyphosphoinositides, the most negatively charged phospholipids known, at specific

sites of the TGN membrane (or other donor membranes) could be crucial for membrane budding and vesicle fission in two (not necessarily mutually exclusive) ways: (1) by directly promoting these processes, both of which involve dramatic changes in membrane leaflet curvature and lipid orientation; (2) by mediating the recruitment, to the donor membrane, of cytosolic proteins required for membrane budding (coat proteins such as ARF (ref. 18 and refs therein) and clathrin adaptors^{19,22}) and vesicle fission (dynamin, perhaps through its pleckstrin homology domain^{23,24}; VPS1 (ref. 25)). Clearly, our finding that PtdIns-TP functions in secretory vesicle formation raises several possibilities¹⁸ of how proteins and lipids may cooperate in budding and fission. □

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A WD-domain protein that is associated with and phosphorylated by the type II TGF- β receptor

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TRANSFORMING growth factor- β (TGF- β) is the prototype for a family of extracellular polypeptides that affect cell proliferation and differentiation, and tissue morphogenesis¹. TGF- β signalling is mediated by two types of serine/threonine kinase receptors, the type I and II receptors, which are able to form a heteromeric complex². No cytoplasmic proteins that associate with these receptors *in vivo*, or are their kinase targets, have yet been described. We have now identified a WD-domain-containing protein, TRIP-1, which specifically associates with the type II TGF- β receptor in a kinase-dependent way. TRIP-1 does not interact with the type II activin or type I receptors, but associates with the heteromeric TGF- β receptor complex. TRIP-1 is phosphorylated on serine and

threonine by the receptor kinase, strongly suggesting that it has a role in TGF- β signalling. This is supported by coexpression of TRIP-1 and type II receptor during development. The existence of TRIP-1 homologues in plant and yeast suggests a conserved function in all eukaryotes.

The type I and type II TGF- β receptors (RI and RII) form homodimers^{3,5} and the receptor complex is probably a heterotetramer of RI and RII homodimers^{2,5}, in which RII is constitutively autophosphorylated and transphosphorylates RI (ref. 6). Both receptors are required for TGF- β -induced growth inhibition and extracellular matrix protein synthesis in Mv1Lu cells⁷, but several findings suggest divergent signalling pathways for the two sets of responses and an association of RII function with TGF- β 's antiproliferative activity^{2,8}.

Using the cytoplasmic domain of RII (RIIC) as 'bait', we performed a yeast two-hybrid screen⁹ of a B-cell library to isolate complementary DNAs of interacting proteins. One clone that scored for specific interaction with RIIC contained a partial cDNA and allowed subsequent isolation of a 1.3 kilobase (kb) cDNA with an open reading frame of 325 amino acids. This protein contains five WD domains¹⁰ (Fig. 1a) and was named TRIP-1 (for TGF- β -receptor interacting protein-1). *In vitro* translated TRIP-1 interacted specifically with a glutathione-S-transferase (GST)-RIIC fusion protein, but not with GST alone (Fig. 1b). Thus, RIIC and TRIP-1 associate directly. TRIP-1 antiserum reacted specifically with an endogenous protein of relative molecular mass 37,000 (M_r 37K) (Fig. 1c, lane 1) and transfected 293 cells expressing TRIP-1 with a Glu-epitope tag

a

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-37 GGCACGAGGT TCGCGCCTTC CTCGCGTCAC CGCCGGGATG AAGCCGATCC TACTGCAGGG CCATGAGCGG 33
                                     M K P I L L Q G H E R 11

TCCATTACGC AGATTAAGTA TAACCGCGAA GGAGACCTCC TCTTTACTGT GGCCAAGGAC CCTATCGTCA 103
S I T Q I K Y N R E G D L L F T V A K D P I V 34

ATGTATGGTA CTCTGTGAAT GGTGAGAGGC TGGGCACCTA CATGGGCCAT ACCGGAGCTG TGTGGTGTGT 173
N V W Y S V N G E R L G T Y M G H T G A V W C V 58

GGACGCTGAC TGGGACACCA AGCATGTCCT CACTGGCTCA GGTGACAACA GCTGTCTGCT CTGGGACTGT 243
D A D W D T K H V L T G S A D N S C R L W D C 81

GAAACAGGAA AGCAGCTGGC CCTTCTCAAG ACCAATTCGG CTGTCCGGAC CTGCGGTTTT GACTTTGGGG 313
E T G K Q L A L L K T N S A V R T C G F D F G 104

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G N I I M F S T D K Q M G Y Q C F V S F F D L R 128

GGATCCGAGC CAGATTGACA ACAATGAGCC CTACATGAAG ATCCCTTGCA ATGACTCTAA AATCACCAGT 453
D P S Q I D N N E P Y M K I P C N D S K I T S 151

GCTGTTTGGG GACCCCTGGG GGAGTGCATC ATCGCTGGCC ATGAGAGTGG AGAGCTCAAC CAGTATAGTG 523
A V W G P L G E C I I A G H E S G E L N Q Y S 174

CCAAGTCTGG AGAGGTGTTG GTGAATGTTA AGGAGCACTC CCGGCAGATC AAGGACATCC AGTTATCCAG 593
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D M T M F V T A S K D N T A K L F D S T T L E 221

CATCAGAAGA CTTTCCGGAC AGAACGTCCT GTCAACTCAG CTGCCCTCTC CCCCAACTAT GACCATGTGG 733
H Q K T F R T E R P V N S A A L S P N Y D H V 244

TCCTGGGCGG TGGTCAGGAA GCCATGGATG TAACCACAAC CTCCACCAGG ATTGGCAAGT TTGAGGCCAG 803
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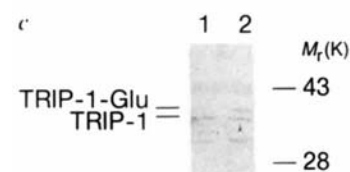
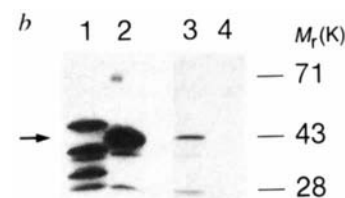
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F F H L A F E E E F G R V K G H F G P I N S V 291

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A F H P D G K S Y S S G G E D G Y V R I H Y F 314

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D P Q Y F E F E F E A *

CCTGTAATCC CACCCTTTT TTITAAGGCA GCGGATCAC CTGAGGTCAG GAGTTTAAGA CCAGCCTGAC 1083
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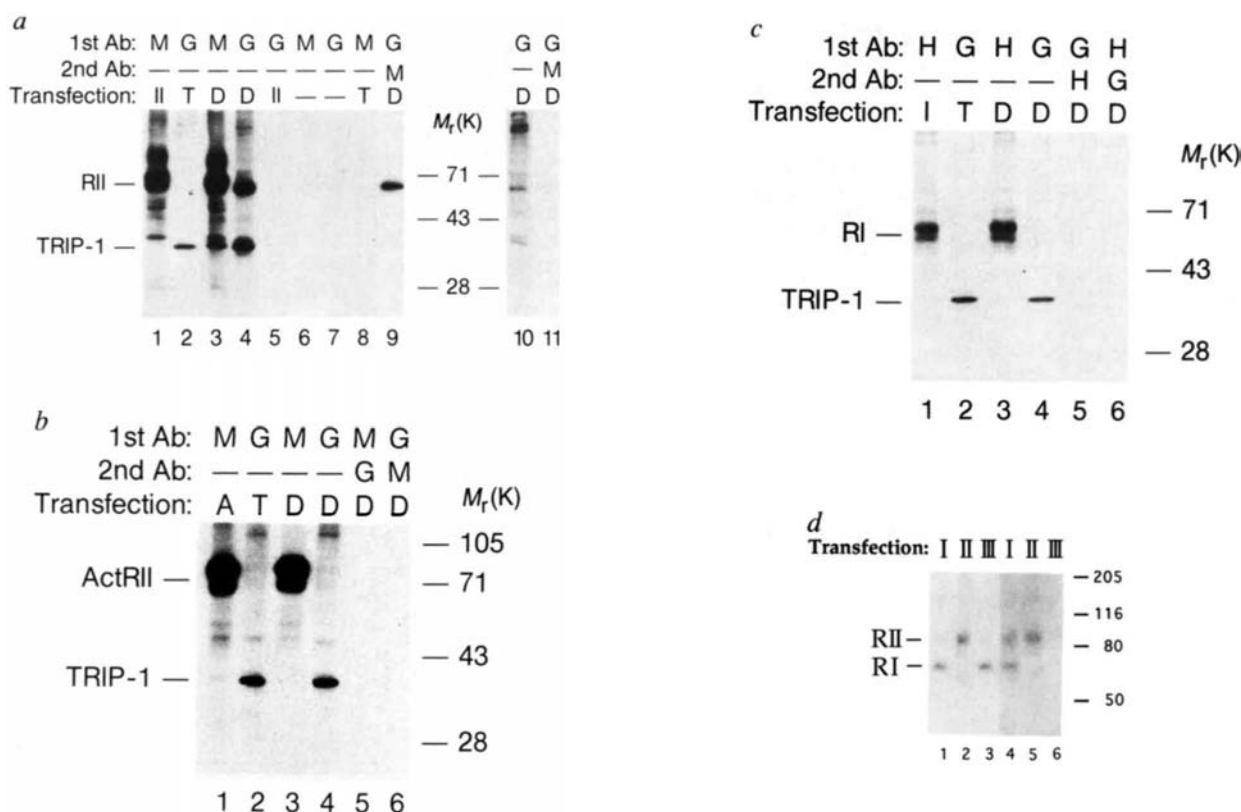


FIG. 1 *cDNA and polypeptide sequences of TRIP-1, in vitro association of TRIP-1 with RIIC and TRIP-1 expression in 293 cells.* **a**, Nucleotide and amino-acid sequences of TRIP-1. The WD domains and a putative ATP/GTP binding site are underlined with solid and dashed lines, respectively. The stop codon is marked with an asterisk. **b**, Interaction of TRIP-1 with the cytoplasmic domain of RII *in vitro*. *In vitro* translated, ^{35}S -labelled TRIP-1 fused to the GAL4 activation domain (GAL4-TRIP-1) was analysed by SDS-PAGE (lane 2). The 43K band (arrow) is GAL4-TRIP-1, whereas the smaller bands may result from internal initiation or degradation. A ^{35}S -labelled fusion of GAL4 with an unrelated protein serves as control for the translation specificity (lane 1). ^{35}S -labelled GAL4-TRIP-1 was incubated with GST-RIIC (lane 3) or GST (lane 4) and the absorbed proteins were separated by SDS-PAGE. The GAL4 activation domain did not interact with GST-RIIC (data not shown). **c**, Expression of endogenous TRIP-1 and transfected Glu-tagged TRIP-1 in 293 cells. Lysates of untransfected (lane 1) or transfected cells expressing Glu-tagged TRIP-1 (lane 2) were analysed in western blot using affinity-purified TRIP-1 antiserum against peptide 127–141. Pre-immune serum did not detect TRIP-1 (data not shown). **METHODS.** Plasmid pAScRII encoding human RIIC fused to the 3' end of the GAL4DNA-binding domain²¹, was used for two-hybrid screening^{9,22} of a human B-lymphocyte cDNA library in yeast Y190. Resulting clones were mated with Y187 strains containing the unrelated proteins SNF1, lamin, p53 and CDC2 fused to the GAL4 DNA-binding domain and tested for β -galactosidase activity. Among 8×10^5 transformants, 41 colonies interacted specifically with RIIC. A positive clone, pACTB18, provided a partial TRIP-1 cDNA. The 5' cDNA sequence was obtained by screening²³ a placenta library with the p-ACTB18 cDNA and PCR cloning of cDNAs from several libraries. To evaluate binding of ^{35}S -labelled TRIP-1 to RIIC, we generated pGEX-IIC, a derivative of pGEX2TK²⁴, which expresses a fusion of GST and RIIC (amino acids 204–567) in *E. coli*. To generate ^{35}S -labelled TRIP-1, a T7 promoter was inserted by PCR 5' of the GAL4-TRIP-1 sequence, and this fragment was subjected to *in vitro* transcription/translation (Promega). Binding of ^{35}S -proteins to purified GST-RIIC or GST was in 50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 2 mM EDTA, 0.1% NP40, 1 mM PMSF, 5 $\mu\text{g ml}^{-1}$ leupeptin, 5 $\mu\text{g ml}^{-1}$ aprotinin for 1 h at 4 °C. pTRIP-1G which expresses Glu-epitope (MGEEETMPME) tagged TRIP-1 in transfected²⁵ 293 cells was derived from pRK5 (ref. 26).

FIG. 2 *In vivo association of TRIP-1 with RII and RI-RII heteromeric TGF- β receptor complex but not with RI or activin type II receptor.* **a**, Association of TRIP-1 with RII. 293 cells were transfected with 10 μg of expression plasmid for Myc-tagged RII (II) or Glu-tagged TRIP-1 (T), or with 10 μg of each plasmid (D; lanes 3, 4, 9), or 2 μg of each plasmid together with 16 μg of carrier DNA (D; lanes 10, 11). ^{35}S -lysates were subjected to single (lanes 1–8, 10) or two sequential (lanes 9, 11) immunoprecipitations using the antibodies indicated (M, anti-Myc; G, anti-Glu). TRIP-1 coprecipitated with RII (lane 3), and RII coprecipitated with TRIP-1 (lanes 4, 9). Lanes 10 and 11 are similar to lanes 4 and 9, except that the expression of RII and TRIP-1 are much lower; RII expression is, based on ^{125}I -TGF- β -receptor crosslinking, similar to endogenous RII levels in C2C12 cells (data not shown). The band above the RII mark is a glycosylated RII form⁴. The higher expression of RII than of TRIP-1 (lanes 1 and 2) results in a lower intensity of TRIP-1 than of RII. RII and TRIP-1 also coimmunoprecipitated in transfected COS-1 cells (data not shown). **b**, TRIP-1 does not associate with the type II activin receptor. Transfected 293 cells expressed Myc-tagged activin type IIA receptor (A) or Glu-tagged TRIP-1 (T) or both (D). ^{35}S -lysates were used for single (lanes 1–4) or two sequential (lanes 5, 6) immunoprecipitations (M, anti-Myc; G, anti-Glu). The receptor (ActRII) runs as a double band, presumably because of glycosylation differences. **c**, TRIP-1 does not associate with RI in the absence of RII. Transfected 293 cells expressed HA-tagged RI Tsk7L (I) or Glu-tagged TRIP-1 (T) or both (D). ^{35}S -lysates were used for single (lanes 1–4) or sequential (lanes 5, 6) immunoprecipitations (H, anti-HA; G, anti-Glu). RI runs as a double band owing to glycosylation differences²¹. **d**, TRIP-1 interacts with the RI-RII heteromeric complex. 293 cells were triple-transfected with expression plasmids for RI ALK-5, RII and Glu-tagged TRIP-1 (I) or double-transfected with plasmids for RII and tagged TRIP-1 (II), or for RI and RII (III). Cell surface receptors were crosslinked to ^{125}I -TGF- β and analysed by SDS-PAGE before (lanes 1–3) or after immunoprecipitations using the TRIP-1-specific anti-Glu antibody (lanes 4–6). The lower intensity of the ^{125}I -TGF- β -crosslinked RII in lane 1 compared with lane 2 reflects the inhibition of ^{125}I -TGF- β crosslinking to RII when coexpressed with RI (ref. 12). TRIP-1 associates with the heteromeric RI-RII complex (lane 4) and RII (lane 5). **METHODS.** Expression plasmids for the Myc-tagged RII and HA-tagged RI Tsk7L have been described^{4,21}. Plasmid pActRII-myc, derived from pRK5²⁶, expresses the human type IIA activin receptor¹³ with a C-terminal Myc tag. Transfection²⁵, metabolic labelling^{4,21}, ^{125}I -TGF- β -1-crosslinking²⁷ and immunoprecipitations^{4,21} were done as described.

contained an additional 38.5K band (Fig. 1c, lane 2), the size expected for tagged TRIP-1. Considering the 30–50% transfection efficiency, the expression levels of tagged TRIP-1 did not greatly exceed endogenous levels; thus, any association of transfected TRIP-1 should not be attributed to overexpression.

To evaluate the association of TRIP-1 with RII *in vivo*, we expressed Myc-tagged RII and Glu-tagged TRIP-1 in 293 cells, which lack detectable endogenous RII (refs 11, 12). TRIP-1 coimmunoprecipitated with RII (Fig. 2a, lane 3) and vice versa (Fig. 2a, lanes 4, 9). The similar intensity of RII and TRIP-1 in anti-Glu immunoprecipitations (lane 4) suggests equal stoichiometry. TRIP-1 also associated with RII at levels comparable to endogenous receptor levels in C2C12 cells (Fig. 2a, lanes 10, 11). Finally, Myc-tagged TRIP-1 also associated with Flag-tagged RII, demonstrating that the association was independent of epitope tags. No difference in association was observed between TGF- β -treated and untreated cells (data not shown).

In contrast to RII, the related type IIA activin receptor¹³ did not coimmunoprecipitate with TRIP-1 (Fig. 2b), nor did TRIP-1 interact with RI Tsk7L (refs 11, 12) (Fig. 2c). Furthermore, TRIP-1 did not interact with RI Tsk7L or ALK-5 (ref. 14) in two-hybrid experiments. Thus, TRIP-1 association is probably specific for RII. Finally, ligand-bound RI–RII heteromeric complex could be coimmunoprecipitated with TRIP-1 (Fig. 2d).

Mutation of lysine at position 277 to arginine abolishes the kinase and signalling activities of RII (refs 6, 7). In contrast to wild-type RII (Fig. 2a; Fig. 3a, lane 6), kinase-defective RII did not efficiently coimmunoprecipitate with TRIP-1 (Fig. 3a, lane 8) and vice versa (Fig. 3a, lane 7). Furthermore, TRIP-1 did not interact with kinase-defective RIIc in two-hybrid experiments. Thus, association of TRIP-1 with RII requires RII's kinase activity, suggesting that receptor autophosphorylation creates a binding site for TRIP-1. Following immunoprecipitation, TRIP-1 was ³²P-phosphorylated *in vitro* in the presence (Fig. 3b, lane

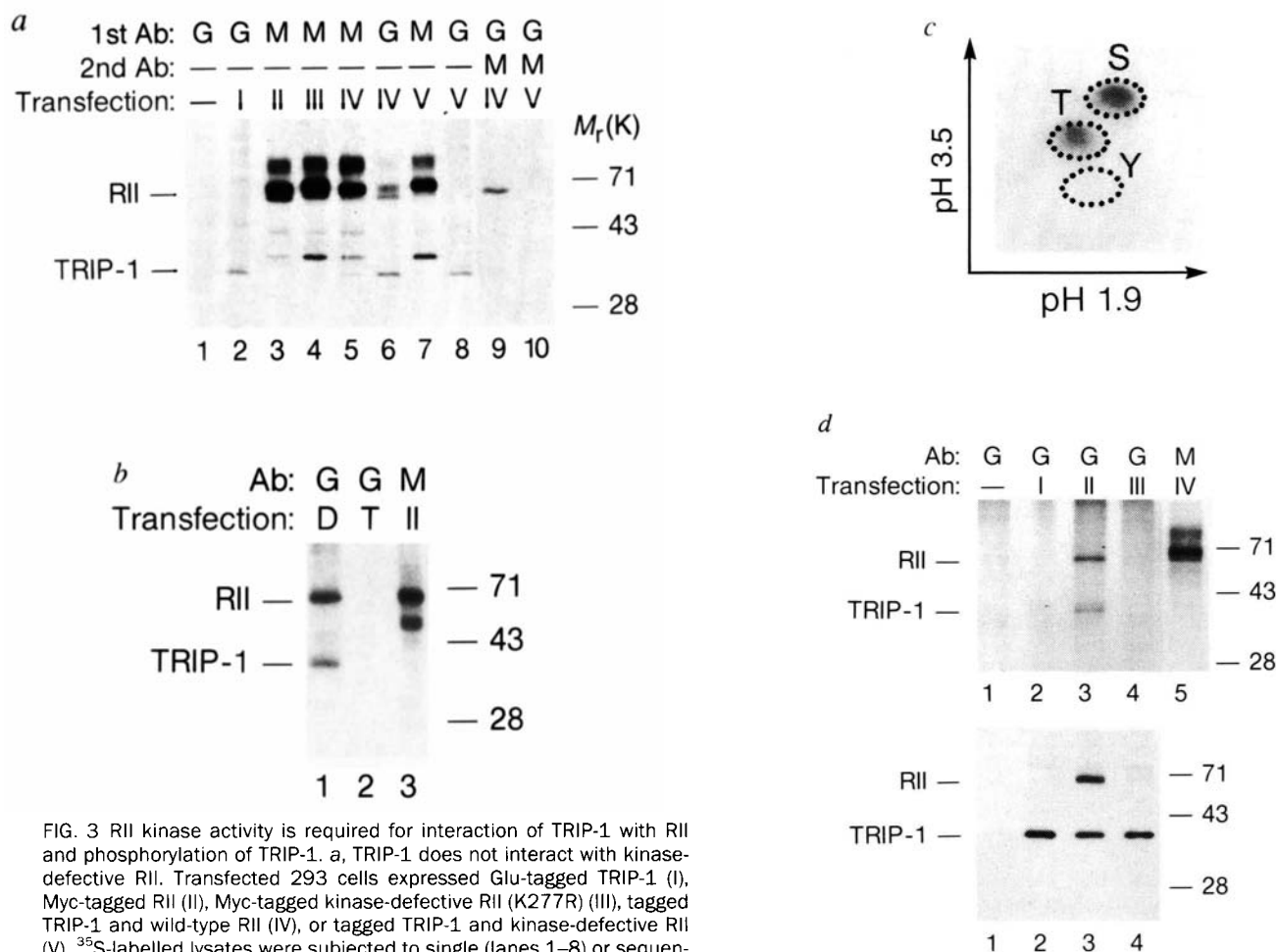


FIG. 3 RII kinase activity is required for interaction of TRIP-1 with RII and phosphorylation of TRIP-1. **a**, TRIP-1 does not interact with kinase-defective RII. Transfected 293 cells expressed Glu-tagged TRIP-1 (I), Myc-tagged RII (II), Myc-tagged kinase-defective RII (K277R) (III), tagged TRIP-1 and wild-type RII (IV), or tagged TRIP-1 and kinase-defective RII (V). ³⁵S-labelled lysates were subjected to single (lanes 1–8) or sequential (lanes 9, 10) immunoprecipitations (M, anti-Myc; G, anti-Glu). The kinase-defective RII runs slightly slower than wild-type RII. Wild-type RII (lanes 6, 9) but not kinase-defective RII (lanes 8, 10) coimmunoprecipitated with TRIP-1. Accordingly, TRIP-1 coprecipitated with wild-type RII (lane 5), but not kinase-defective RII (lane 7). **b**, *In vitro* phosphorylation of TRIP-1 by the immunoprecipitated RII complex. Transfected 293 cells expressed Myc-tagged RII (II) or Glu-tagged TRIP-1 (T) or both (D). Immunoprecipitates were subjected to *in vitro* kinase reactions and ³²P-labelled proteins were separated by SDS-PAGE. TRIP-1 is phosphorylated when coexpressed with RII (lane 1), but not in its absence (lane 2). In control experiments, RII is autophosphorylated (lane 3). The band below RII (lane 3) is a frequently observed degradation product of RII. **c**, TRIP-1 is phosphorylated on serine and threonine, but not on tyrosine. ³²P-labelled TRIP-1 (**a**, lane 1) was subjected to phosphoamino acid analysis using two-dimensional thin-layer electrophoresis. The positions of co-electrophoresed unlabelled phosphoamino acids are indicated

(T, threonine; S, serine; Y, tyrosine). **d**, *In vivo* phosphorylation of TRIP-1. Transfected 293 cells expressed Glu-tagged TRIP-1 (I) or Myc-tagged RII (IV) or both (II), or tagged TRIP-1 and Myc-tagged kinase-defective RII (III). Cells were metabolically labelled with ³²P-orthophosphate (top panel) or ³⁵S-methionine/cysteine (bottom panel) and subjected to immunoprecipitation. TRIP-1 associates with wild-type RII and is phosphorylated (lanes 3). In contrast, TRIP-1 by itself (lanes 2) or in the presence of kinase-defective RII (lanes 4) is not phosphorylated and does not associate with kinase-defective RII.

METHODS. Plasmid pRII(K277R)-myc expresses Myc-tagged human RII with a K277R mutation. In parallel with ³⁵S-labelling^{4,21}, transfected²⁵ cells were labelled with 1 mCi ml⁻¹ ³²P-orthophosphate (NEN). For *in vitro* kinase assay, immunoprecipitates bound on protein A-Sepharose were incubated in 20 μ l of 25 mM HEPES, pH 7.5, 10 mM MnCl₂, 10 μ M ATP and 10 μ Ci [γ -³²P]ATP (Amersham) at 25 °C for 15 min. Phosphoaminoacids were analysed as described²⁸.

1) but not in the absence of RII (Fig. 3b, lane 2). Its phosphorylation on serine and threonine (Fig. 3c) is consistent with the kinase specificity of RII. No other proteins were detected in anti-Glu immunoprecipitations (Fig. 3d), supporting a direct phosphorylation of TRIP-1 by associated RII. Finally, TRIP-1 was also phosphorylated in intact cells expressing kinase-active RII (Fig. 3d). Taken together, our results strongly suggest that TRIP-1 is a substrate of the RII kinase *in vivo*.

If TRIP-1 is required for RII signalling, we would expect coexpression of both proteins in various tissues. All tissues tested expressed a 1.6-kb TRIP-1 messenger RNA (Fig. 4a) and the pattern of TRIP-1 immunoreactivity in embryonic day (E) 14.5

mouse embryos correlated well with RII staining (Fig. 4b), further supporting a functional correlation between both proteins.

The association with and phosphorylation by RII strongly suggest that TRIP-1 represents an early step in TGF- β receptor signalling. Its ligand-independence is consistent with the constitutive homomeric complex formation^{3,5} and autophosphorylation⁶ of RII, and may resemble the constitutive association of TRAFs with the TNF receptor¹⁵ and CD40 (ref. 16), and of Jak kinases with cytokine receptors¹⁷. Many WD proteins interact with multiple proteins¹⁸, and WD domains may present a conformation-sensitive, changeable surface in protein-

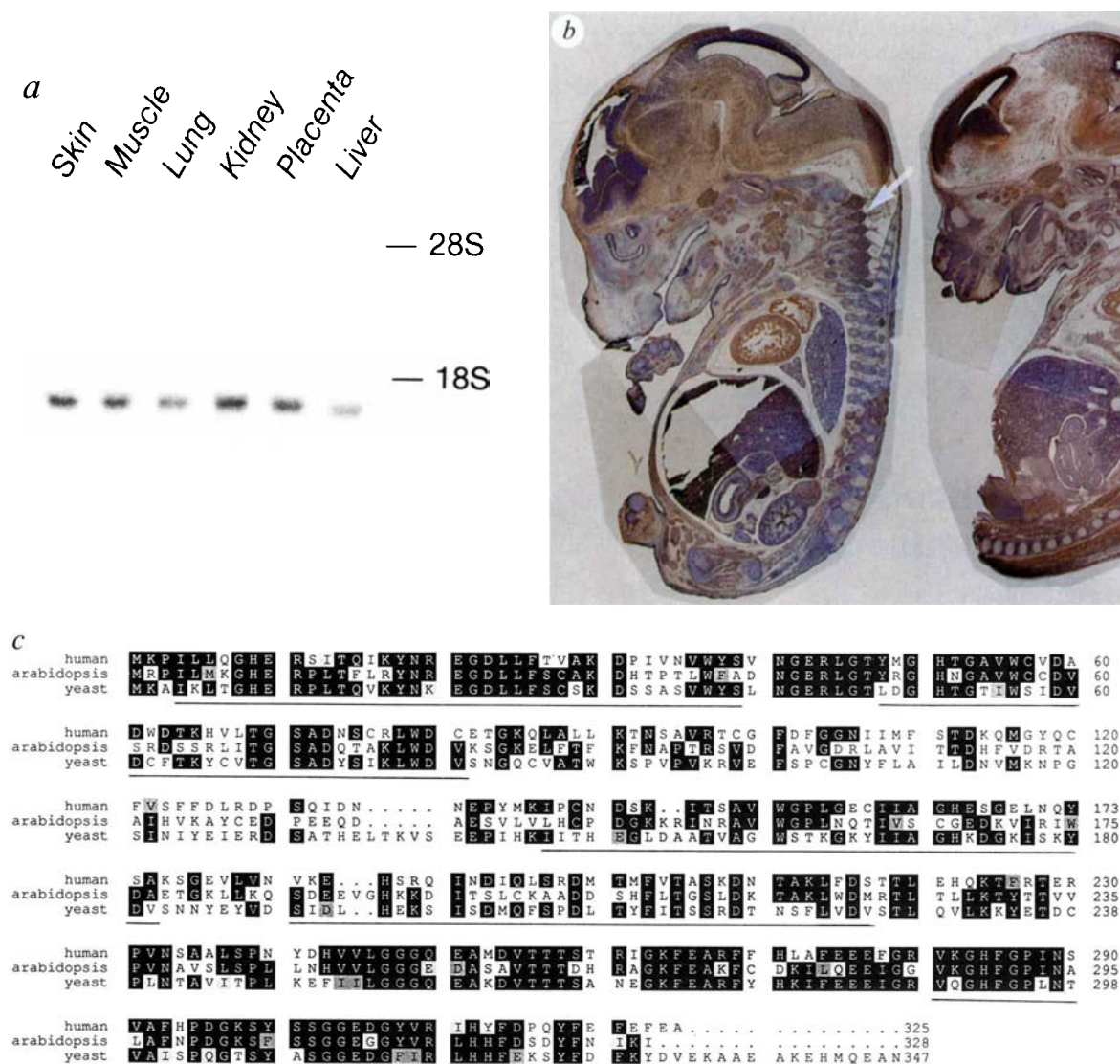


FIG. 4 Expression of TRIP-1 in human fetal tissues and colocalization with RII in E14.5 mouse embryo. Sequence alignment of TRIP-1 with its yeast and plant homologues. **a**, Equal amounts of RNA (30 μ g) were hybridized with ³²P-labelled TRIP-1 cDNA. A single TRIP-1 mRNA species of 1.6 kb is detected. **b**, Sections of E14.5 mouse embryos were immunostained with affinity-purified anti-TRIP-1 (left) and anti-RII (right); brown colour shows immunoreactivity. Both TRIP-1 and RII are immunolocalized in epithelial, mesenchymal and neuronal derivatives (for example, spinal ganglia; arrow). TRIP-1 expression was apparent in skin and whisker follicles, tongue, thyroid gland, brain including choroid plexus, cochlea, spinal cord, muscular layer of the intestine, periosteum, hypertrophic chondrocytes and spinal ganglia. Branching tubules of lung and kidney and the seminiferous tubules of the testes (data not shown) also showed TRIP-1 immunoreactivity. Heart stained intensely with anti-

TRIP-1 except for the valves. Specificity controls using consecutive sections, that is, omitting the primary antisera or replacing it with normal rabbit sera or unrelated antisera, gave no staining (data not shown). **c**, Sequence comparison of human TRIP-1 and its homologues in *A. thaliana* and *S. cerevisiae*. Dots represent gaps for optimal alignment. Identical amino acids are in black boxes; conservative substitutions are in grey boxes and WD domains are underlined.

METHODS. Human fetal tissue northern blots were hybridized under high-stringency conditions using a ³²P-labelled TRIP-1 cDNA probe. Mouse E14.5 embryos were processed to paraffin sections by routine procedures. Immunostaining using anti-TRIP-1 antiserum (Fig. 1c) and rabbit anti-RII (Santa Cruz Biotechnology; 1–10 μ g ml⁻¹) was done as described²⁹. The cDNA for the *A. thaliana* TRIP-1 homologue was cloned by standard PCR procedures starting from an *Arabidopsis* cDNA library.

protein interactions¹⁰. Thus, the role of TRIP-1 may involve a conformational change allowing recruitment of downstream proteins or its phosphorylation by RI in the RI-RII complex. The function of TRIP-1 may be analogous to GRB-2, which recruits signalling components to receptor tyrosine kinases.¹⁹

Considering the similarity in activities of activin and TGF- β and structures of their type II receptors, the lack of TRIP-1 association with activin RII suggests the existence of TRIP-1-related proteins for the related type II receptors. Thus, TRIP-1 may define a class of WD proteins involved in signalling by TGF- β superfamily members. Homology searches and polymerase chain reaction (PCR)-based cDNA cloning revealed a TRIP-1-related gene in *Arabidopsis thaliana* and *Saccharomyces cerevisiae*. All three sequences are of similar length and have five WD repeats at corresponding positions and 39–46% amino-acid identity, including flanking regions between WD domains (Fig. 4c). These features suggest that these proteins are homologues. In *S. cerevisiae*, the induction of G1 arrest by α -factor²⁰ has similarities with the growth arrest by TGF- β . Studies of yeast TRIP-1 may shed light on the mechanism of TGF- β signal transduction. □

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Radiation-induced cell cycle arrest compromised by p21 deficiency

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THE protein p21 is a dual inhibitor of cyclin-dependent kinases^{1–3} and proliferating-cell nuclear antigen (PCNA)⁴, both of which are required for passage through the cell cycle. The p21 gene is under the transcriptional control of p53 (ref. 5), suggesting that p21 might promote p53-dependent cell cycle arrest or apoptosis. p21 has also been implicated in cell senescence⁶ and in cell-cycle withdrawal upon terminal differentiation^{7–9}. Here we investigate the role of p21 in these processes using chimaeric mice composed partly of p21^{-/-} and partly of p21^{+/+} cells. Immunohistochemical studies of the p21^{+/+} and p21^{-/-} components of adult small intestine indicated that deletion of p21 had no detectable effect on the migration-associated differentiation of the four principal intestinal epithelial cell lineages or on p53-dependent apoptosis following irradiation. However, p21^{-/-} mouse embryo fibroblasts are impaired in their ability to undergo G1 arrest following DNA damage.

To investigate the many proposed functions of p21, a null allele was generated in 129/Sv embryonic stem (ES) cells by replacing the p21 coding sequence with a neomycin-resistance cassette (*neo*^r; Fig. 1a–c). Homozygous p21^{-/-} ES cells were produced by subjecting two heterozygous p21^{+/-} cell lines to selection in an increased concentration of G418. One p21^{-/-} ES cell clone was recovered from each parental p21^{+/-} line. p21 deficiency was verified by Southern blot analysis (Fig. 1b, c) and by immunoprecipitation of ES cell lysates with a p21 polyclonal antiserum (data not shown).

The small intestine was chosen as a model for assessing the role of p21 in proliferation, differentiation and cell death¹⁰. Studies of adult aggregation chimaeras indicate that each intestinal crypt is monoclonal whereas each villus receives cells from 2–3 surrounding crypts. Introduction of 129/Sv ES cells, such as those lacking p21, into normal B6 blastocysts results in B6 $\leftrightarrow$ 129/Sv chimaeras that contain patches of B6 crypt-villus units and patches of 129/Sv crypt-villus units. These can be readily distinguished on the basis of differences in their ability to bind the lectin UEA-1 (ref. 11). In B6 $\leftrightarrow$ 129/Sv chimaeras, proliferation was restricted to undifferentiated epithelial cells in both p21^{+/+} and p21^{-/-} crypts (Fig. 2a, c). Furthermore, multi-label immunohistochemical surveys using a well characterized panel of antibodies and lectins¹¹ revealed that loss of p21 had no detectable effects on the ability of the intestine to complete normal morphogenesis or to maintain the differentiation programs of four principal epithelial cell lineages (for example, see Fig. 2f). Low to moderate doses of γ -irradiation cause cell cycle arrest and p53-dependent apoptosis in crypts^{12–14}. Irradiation caused the same 100–150-fold increase in the frequency of cell death in crypts composed either of p21^{+/+} or of p21^{-/-} cells (Fig. 2g, h), indicating that p21 is not required for p53-dependent cell death, at least in this system. p21^{-/-} and p21^{+/+} crypts also showed a similar reduction in 5-bromodeoxyuridine (BrdU)-positive cells following irradiation. Thus, radiation-induced cell cycle arrest occurred in undifferentiated intestinal epithelial cells regardless of p21 status (Fig. 2d, e). However, as cell cycle arrest in these cells has not been shown to require p53,

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we examined the role of p21 in DNA-damage-induced G1 arrest in fibroblasts, which is p53 dependent.

Cultures of primary mouse embryo fibroblasts (MEFs) were prepared from B6 $\leftrightarrow$ 129/Sv $p21^{-/-}$ chimaeras produced from two different $p21^{-/-}$ ES cell lines (p21 A and p21 B) and from B6 $\leftrightarrow$ 129/Sv $p53^{-/-}$ chimaeras. MEFs were also prepared from wild-type embryos and from chimaeras generated from an ES cell line carrying a random integration of the $p21$ -targeting vector. Initial MEF cultures were 30–70% 129/Sv (Fig. 3a). Highly pure MEF populations (>95% 129/Sv) were obtained following G418 selection (Fig. 3a). It should be noted that this enrichment required no more than two population doublings. The absence of p21 protein in the $p21^{-/-}$ MEFs was confirmed by immunoprecipitation (Fig. 3b, c), and the purity of p21-deficient cell populations was verified by immunofluorescence with a p21-specific antiserum (data not shown).

Immunoprecipitation with anti-CDC2 -CDK2 or -CDK6 antibodies showed that the pattern of cyclin-CDK complexes was indistinguishable in the $p21^{-/-}$ and $p53^{-/-}$ MEFs, although the $p21^{-/-}$ MEFs still contain wild-type p53, as judged by immunoprecipitation with conformation-specific anti-p53 antibodies (Fig. 3d). In these cells, p21 was absent from CDK2 and from CDC2 complexes, and CDK6 was primarily associated with p16 (Fig. 3c). In contrast to $p53^{-/-}$ MEFs, $p21^{-/-}$ cell populations did not show obvious differences in proliferation rates compared with normal controls (not shown).

The two $p21^{-/-}$ MEF populations, together with $p53^{-/-}$, random integration and wild-type controls, were treated with γ -radiation and subjected to cell-cycle analysis. The G1-phase radiation response was assessed by comparing the S-phase fraction in irradiated cells (at 18 hours after a dose of 5.5 Gy) to that in untreated cell populations. Wild-type cells and cells

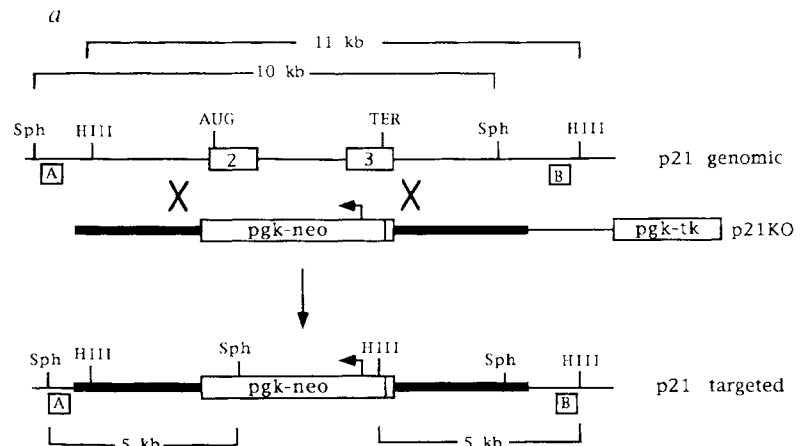


FIG. 1 p21 gene targeting in ES cells. a, Targeting scheme. The targeting vector, p21KO, contains ~8 kb of p21 genomic sequences (thick lines) flanking the pgk-neo expression cassette which is located in the opposite transcriptional orientation to p21. Recombination on the 5'-side of p21 was monitored by Southern blotting using an *Sph*I digest and probe A. The 3' recombination junction was characterized by a *Hind*III digest of genomic DNA and probe B. Both probes lie outside the genomic regions present in the targeting vector. b and c, Southern blot analysis of ES cell clones. DNA from the parental D3 ES cells, two heterozygous mutant clones ($p21^{+/-}$ A and B) and two homozygous mutant clones that were derived from the heterozygous clones following exposure to increased G418 concentrations ($p21^{-/-}$ A and B) are shown. Using probe A on *Sph*I-digested genomic DNA (b), the $p21^{+/-}$ clones show a ~5 kb mutant-specific band (band 'd') in addition to the ~10-kb band (band 'w') corresponding to the wild-type p21 allele. In the $p21^{-/-}$ cell lines the wild-type band is absent. At the 3' recombination junction (*Hind*III-digested DNA and probe B, in c), $p21^{+/-}$ A shows the expected mutant-specific band of ~5 kb (band d); the mutant band in clone B is ~3.6 kb (band d*). Extensive restriction mapping revealed that $p21^{+/-}$ B contained a 1.4-kb deletion 3' to exon 3 of p21. In both $p21^{-/-}$ lines, the band corresponding to the wild-type allele of p21 is again absent. Gene targeting was performed using D3 ES cells as described¹⁹⁻²¹. $p21^{-/-}$ ES cell clones were generated from $p21^{+/-}$ ES cells by selection with 1.8 mg ml⁻¹ G418 (active weight) for 10–11 days.



carrying the random integration were substantially arrested in G1 following irradiation (Fig. 4a). The fraction of cells in S phase after irradiation was 39.5 or 39%, respectively, of that in non-irradiated cells. For $p53^{-/-}$ MEFs, the S-phase fraction after irradiation was 84.5% of the value in untreated cells, a result that is consistent with previous reports demonstrating that p53 is required for G1 cell-cycle arrest following DNA damage in fibroblasts¹⁵. The MEF populations derived from the two $p21^{-/-}$ ES cell clones showed an intermediate irradiation response, with post-irradiation S-phase fractions at 57.5 or 54.5% of the level in non-irradiated samples. Similar results were obtained in six independent experiments (Fig. 4a, b).

Several models could explain the intermediate phenotype of $p21^{-/-}$ MEFs. For example, $p21^{-/-}$ cells may differ from wild-type cells in the timing of the radiation response or in the amount

of damage required to induce arrest. We therefore examined the radiation response of $p21^{-/-}$ MEFs at several times following treatment (14, 18 and 22 hours after a dose of 5.5 Gy) and at two different radiation doses (5.5 and 11 Gy, analysed 18 hours after irradiation). In each case, our results were similar to those shown in Fig. 4a, b. The intermediate phenotype of $p21^{-/-}$ cells could alternatively reflect the fact that primary MEFs are composed of a mixture of cell types, each of which may depend to a different extent on p21 for growth arrest in the presence of DNA damage. Finally, the position of cells in G1 phase could determine sensitivity to the p21-dependent mechanism of p53-induced cell-cycle arrest.

It is well established that, following irradiation, the activity of CDK2 kinase is reduced by ~3–5-fold in human fibroblasts¹⁶. The same is true in normal MEFs (Fig. 4c: ~5-fold reduction). However, inhibition of CDK2 is not observed upon irradiation

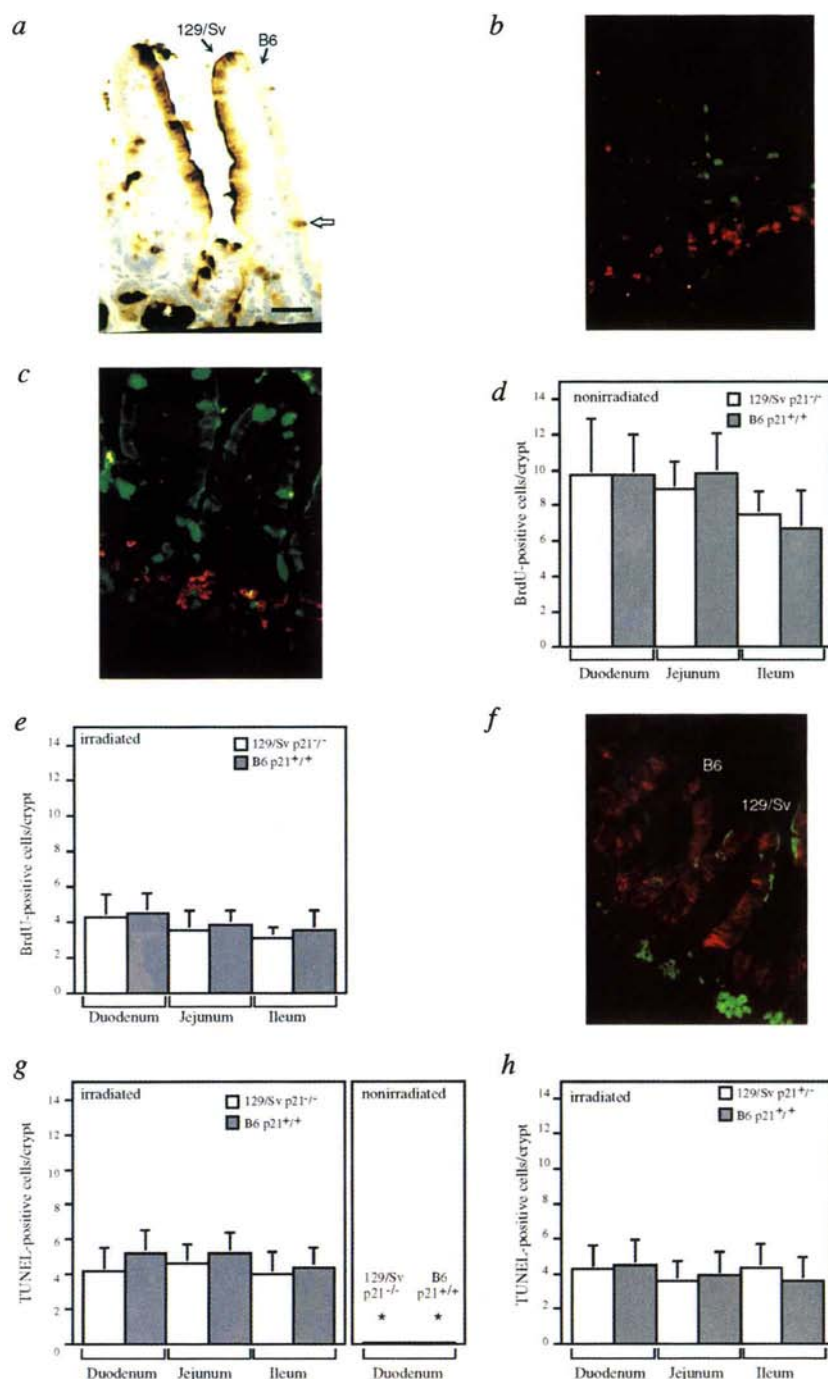


FIG. 2 Use of B6→129/Sv $p21^{-/-}$ chimaeric mice to assess the role of p21 in proliferation, differentiation and death programs. **a**, A polyclonal jejunal villus from a chimaeric mouse stained with peroxidase-conjugated UEA-1. 129/Sv but not B6 enterocytes bind the lectin. A subset of B6 and 129/Sv goblet cells are also labelled (open arrow). **b**, **c**, Adjacent patches of $p21^{+/+}$ B6 and $p21^{-/-}$ 129/Sv jejunum (**b** and **c**, respectively) were stained with FITC-conjugated UEA-1 (green) and anti-BrdU (red). **d**, **e**, S-phase cells were quantitated in crypts from irradiated and non-irradiated (panels **c** and **d**) chimaeras following staining with UEA-1 and anti-BrdU. 30 crypts were counted per section per region per mouse ($n=3$ mice each). Mean values ± 1 s.d. are plotted. **f**, Adjacent B6 and 129/Sv villi show similar patterns of expression of liver fatty-acid-binding protein (L-FABP), a differentiation marker for the enterocytic lineage. The section was stained with FITC-UEA-1 (green) and rabbit anti-L-FABP sera (red). **g**, **h**, Quantitation of p53-dependent apoptosis in crypts from irradiated animals is shown (TUNEL assay). Asterisk denotes that non-irradiated age-matched B6→129Sv $p21^{-/-}$ chimaeras contain on average only one TUNEL-positive cell per 30 crypts. Scale bar in **a**, 25 μ m.

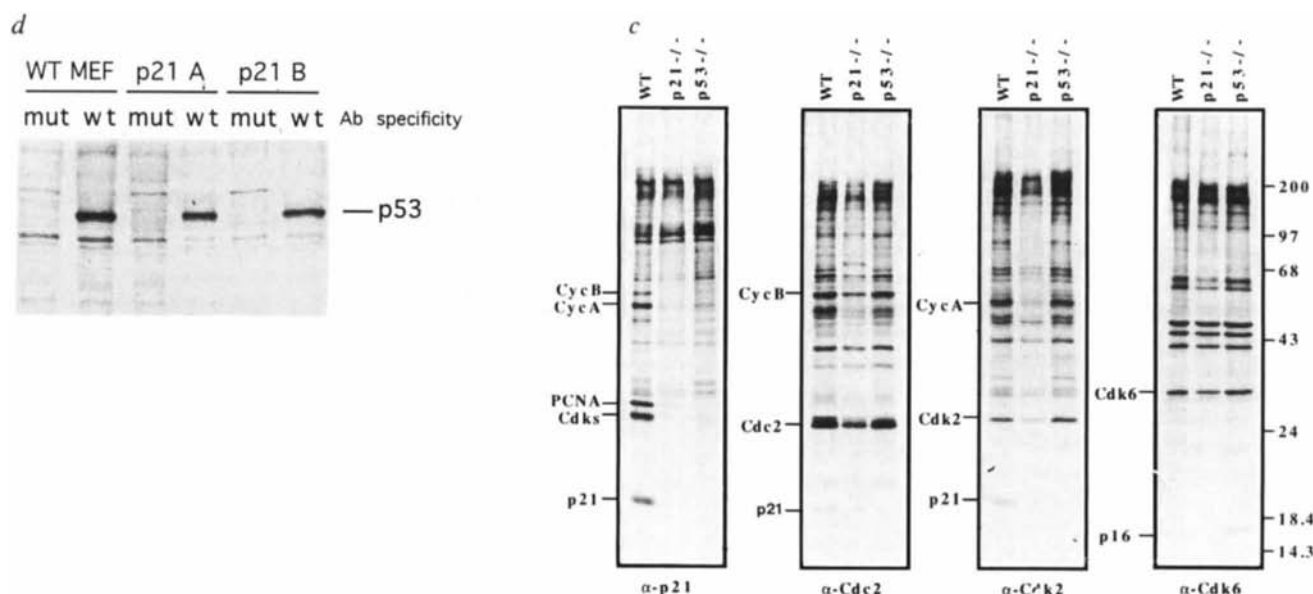
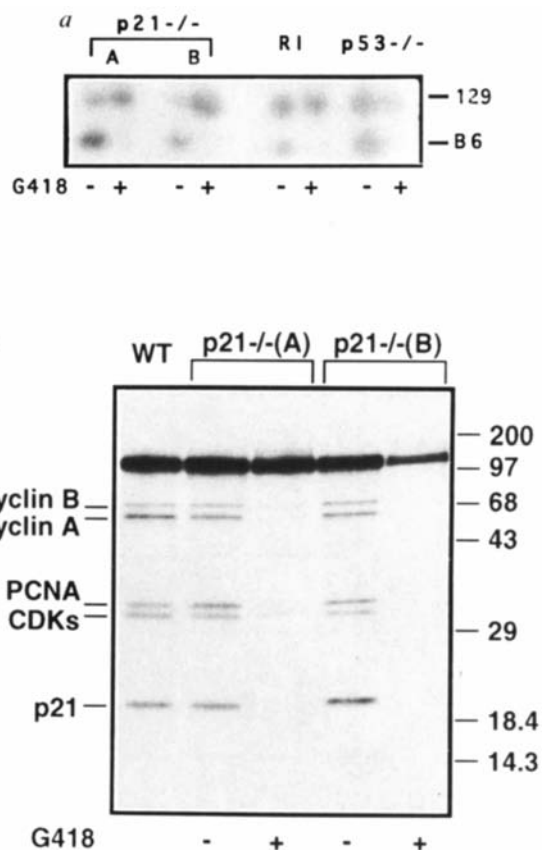
METHODS. Irradiated chimaeras were killed 4.5 h after receiving 6 Gy of total-body γ -irradiation. Fixation and multilabel immunohistochemical staining of chimaeric intestine were as described¹¹. TUNEL assays were performed on periodate-lysine-paraformaldehyde-fixed tissues using the procedure described in ref. 22, except that slides were incubated in 0.5% Triton X-100/PBS for 20 min at room temperature.

of $p53^{-/-}$ or $p21^{-/-}$ cells (Fig. 4c). This demonstrates that DNA-damage-induced inhibition of CDK2 requires p21.

Since its discovery¹⁷, p21 has been implicated in a broad spectrum of biological processes. Inhibition of cell proliferation by p21 has been linked to the permanent cell cycle withdrawal that accompanies senescence⁶. As a transcriptional target of p53, p21 has been proposed as an effector of cell-cycle arrest following DNA damage, as a component of the cell death program and as a tumour suppressor. Our data indicate that p21 is not required for p53-dependent apoptosis (at least in some cell

types). However, the consequences of p21 disruption clearly demonstrate the involvement of this protein in p53-dependent cell-cycle arrest following DNA damage. Our data also indicate that additional p53-responsive growth controls exist. This may help to explain the lack of p21 mutations in human tumours¹⁸. We have followed a small number of chimaeric $p21^{-/-}$ mice (up to 80% $p21^{-/-}$, as judged by coat colour) for more than one year. The fact that none of these animals appears predisposed to cancer provides further evidence against a role for p21 as a major tumour suppressor. Several studies have also suggested a

FIG. 3 Selection of targeted mouse embryo fibroblasts (MEFs). **a**, glucose-6-phosphate isomerase (GPI) isoenzyme analysis. Donor ES cells (strain 129/Sv) contain a GPI isoenzyme with a different electrophoretic mobility from that of the recipient C57BL/6 blastocysts. The ratio of these isoforms was used to estimate the contribution of the targeted and recipient cells to MEF populations. GPI analysis is shown for the initial MEF preparations from representative chimaeras generated from each ES cell clone (as indicated; RI, random integration) and for MEFs enriched for cells derived from targeted ES cells by selection with G418. **b**, $p21^{-/-}$ MEFs lack p21 protein. MEF populations (as indicated) were tested for the presence of p21 by immunoprecipitation. Note that p21 and associated proteins are present in the unselected $p21^{-/-}$ populations but are barely detectable following selection. **c**, Cyclin-CDK complexes in $p21^{-/-}$ cells. Cyclin-CDK complexes were recovered from the indicated cell populations using antisera against p21, CDC2, CDK2 and CDK6. The identities of co-precipitated proteins are indicated. **d**, Immunoprecipitation of p53 with conformation-specific antibodies. p53 protein was recovered from lysates of ³⁵S-labelled cells (indicated) by immunoprecipitation with antibodies specific for either the wild-type (WT; PAb 1620 from Oncogene Sciences) or the mutant (mut; PAb 240, Oncogene Sciences) conformations of p53. **METHODS.** Chimaeras were generated by injection of 11–13 donor ES cells into C57BL/6 blastocysts²¹. 11 days after embryo injection and reimplantation ~E13.5 embryos were collected and MEFs isolated as described²³. Passage-3 MEFs were selected with 300 $\mu\text{g ml}^{-1}$ G418 (Gibco BRL) for 6–7 days. p21 antiserum was raised against a GST-p21 fusion and depleted of anti-GST antibodies by affinity purification. Immunoprecipitations were done as previously described¹.



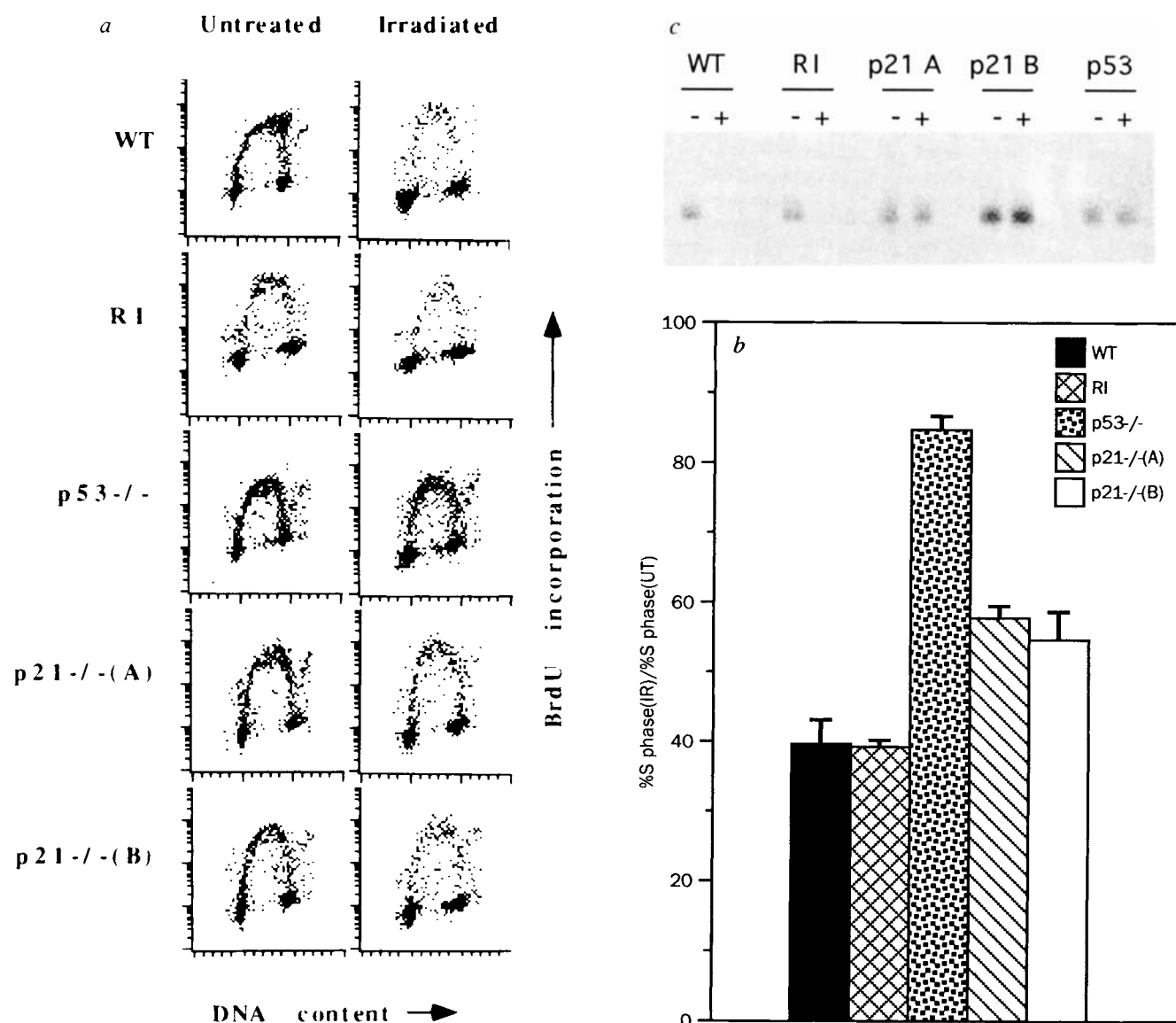


FIG. 4 Radiation response of $p21^{-/-}$ cells. **a**, Representative two-dimensional FACS analysis of untreated and irradiated samples of $p21^{-/-}$, $p53^{-/-}$, random integration (RI) and wild-type (WT) MEFs are shown. Asynchronous cultures were irradiated with a dose of 5.5 Gy and the percentage of cells synthesizing DNA was evaluated with a 4-hour BrdU pulse, beginning 14 hours after irradiation. Cells in the G0 or G1 phase of the cell cycle appear in the lower left quadrant. Cells in the G2 or M phases appear in the lower right quadrant. S-phase cells are positive for BrdU and are detected in the arch that connects G0/G1 and G2/M populations. **b**, Histogram showing the S-phase fraction in irradiated versus untreated samples. A quantitation of the number of cells remaining in S phase following irradiation compared to the number of S-phase cells in an untreated control is shown. The mean of 6 independent experiments is shown for all samples except the random

role for p21 during differentiation in different cell lineages^{7,9}. Our data demonstrate that p21 is not essential for terminal differentiation in the small intestine. Furthermore, we have generated a germline, homozygous p21-deficient animal. The $p21^{-/-}$ mouse is viable and displays no apparent phenotype by seven weeks of age. Thus, the fact that p21 is not solely responsible for p53-dependent growth arrest or cell-cycle withdrawal following differentiation could indicate a general need for tight regulation of cell proliferation by several overlapping pathways.

Note added in proof. Following submission of this paper, Deng *et al.* (*Cell* **82**, 675–684, 1995) reported the generation and char-

acterization of a p21-deficient mouse strain. They also observe that p21 deficient embryo fibroblasts are defective in the irradiation response, while p53-dependent apoptosis and overall embryonic and postnatal development were not affected by the absence of p21.

METHODS. MEFs were grown to subconfluence in DME medium, 10% FCS, 5 mM glutamine with antibiotics. Cells were plated at a density of $5-6 \times 10^5$ cells per 10-cm plate and were irradiated between 24 and 30 h after plating. Cell cycle analysis was as described¹⁵, except that histones were extracted in 0.7% Triton-X 100, 1.2 M HCl. Samples were analysed with a FACScan using CellQuest software (Becton-Dickinson). Immunoprecipitation and histone H1 kinase assays were done as described¹.

acterization of a p21-deficient mouse strain. They also observe that p21 deficient embryo fibroblasts are defective in the irradiation response, while p53-dependent apoptosis and overall embryonic and postnatal development were not affected by the absence of p21.

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Requirement for TFIIF kinase activity in transcription by RNA polymerase II

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AN array of tandem heptapeptide repeats at the carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II constitute a highly conserved structure essential for viability^{1–3}. Studies have established that the CTD is phosphorylated at different stages of the transcription cycle^{4–7}, and that it may be involved in transcriptional regulation^{8–12}. The exact role of the CTD remains elusive, as *in vitro* reconstituted transcription using the adenovirus major late promoter does not require the CTD^{13,14}. Previous studies^{7,15,16} showed that transcription from the murine dihydrofolate reductase (DHFR) promoter can be only accomplished by the form of RNA polymerase II that contains the hypophosphorylated CTD (RNAPIIA), but not by the form that lacks it (RNAPIIB)⁷. Here we show that the CTD, but not its phosphorylation, is required for initiation of transcription. We also show that transcription requires CTD kinase activity provided by the CDK⁷ subunit of TFIIF^{17–19}.

We examined the activity of the A and B forms of RNAPII on the murine dihydrofolate reductase (DHFR) promoter using a reconstituted transcription system. Both preparations of RNAPII (Fig. 1a) exhibited similar specific activities in a run-off transcription assay with the adenovirus major late (AdML) promoter (Fig. 1b,c). However, in agreement with previous studies^{7,15,16}, transcription from the DHFR promoter using the B form of RNAPII was tenfold less efficient in production of full-length transcripts (Fig. 1b, lanes 3 and 6, and c). To identify the step(s) in transcription impaired by the lack of the CTD, we analysed transcription initiation from the DHFR promoter. In the presence of ATP and [α -³²P]CTP, RNAPIIA forms the first phosphodiester bond at the transcriptional start sites on the DHFR and AdML promoters and releases the ApC dinucleotide

as a product of multiple abortive cycles of transcription initiation (Fig. 2a). Analysis of initiation revealed a 15-fold reduction in efficiency of RNAPIIB from the DHFR, but not from the AdML promoter (Fig. 2b, lanes 2 and 4, c). Thus it appears that in the reconstituted transcription system, the requirement for the CTD is related to the steps preceding promoter clearance, these being preinitiation complex formation and/or initiation.

To identify which of these two steps requires the CTD, we used dinucleotide priming²⁰. This technique circumvents the requirement for the formation of the first phosphodiester bond by incorporating dinucleotides corresponding to the transcription start site (Fig. 2d). Dinucleotide priming using the linearized DHFR promoter template resulted in a drastic increase in the activity of RNAPIIB, approaching half that of RNAPIIA (Fig. 2e, lanes 1 and 3, and f). As shown previously²⁰, abortive initiation under these conditions was highly specific. Indeed, it was dependent on TATA-box-binding protein (TBP) (Fig. 2e) and the other general transcription factors (data not shown). Moreover, when ApA was substituted with CpC, no initiation was observed (Fig. 2g). These results indicate that the formation of the first phosphodiester bond during transcription of the DHFR is dependent on the CTD.

Because the CTD is implicated in transcription initiation, a step apparently bypassed by dinucleotide priming, it raises the question of whether productive run-off transcription by RNAPIIB could also be rescued by priming with ApA. A titration of ApA into transcription reactions containing the B form of RNAPII resulted in stimulation of the run-off transcription, but the same titration was without effect on reactions containing the IIA form of RNAP (Fig. 3a). The RNAPIIB activity recovered by dinucleotide priming was only 15% of the RNAPIIA activity (Fig. 3b), which indicates that there must be other critical steps dependent on the CTD during productive transcription. One step may entail the phosphorylation of the CTD²¹. However, a previous study indicated that inhibition of the CTD-kinase activity with the ATP analogue H8 in reconstituted systems transcribing the AdML promoter does not result in transcription inhibition¹⁴. Transcription of the AdML promoter does not discriminate between the A and B forms of RNAPII, so this promoter may not have been the appropriate one with which to test phosphorylation requirements. Indeed, application of H8 resulted in a dose-dependent suppression of transcription from the DHFR gene, but not from the AdML promoter (Fig. 3c). Consistent with results presented above (Fig. 3b), RNAPIIB activity recovered by dinucleotide priming was resistant to H8 inhibition (Fig. 3d). Moreover, reactions containing the dinucleotide ApA and the IIA form of RNAPII were resistant to H8 inhibition (Fig. 3e). It is surprising, however, that under these conditions the levels of transcription were not diminished. It is possible that, in the presence of the dinucleotide, RNAPII enters an 'unusual conformation' which requires the CTD for optimal transcription but bypasses its phosphorylation²². Nonetheless, the above results suggest that transcription from the DHFR promoter requires not only the presence of the CTD, but is also subjected to regulation by phosphorylation.

A CTD kinase is associated with TFIIF^{17,23,24}, its activity residing in the CDK-activating kinase (CAK) composed of Cdk7 and cyclin H^{17–19}. To analyse the role of CAK in transcription from the DHFR promoter, we first tested the effect of affinity-purified antibodies against cyclin H. Incubation of these antibodies with TFIIF before formation of the preinitiation complex abolished both transcription activity and phosphorylation of RNAPII (ref. 19). However, addition of antibodies after the formation of a complete preinitiation complex had no effect on transcription from the AdML promoter¹⁹ (Fig. 4a). In contrast, titration of the cyclin H antibodies on preformed preinitiation complexes using the DHFR promoter strongly suppressed transcription (Fig. 4a). Analysis of the electrophoretic mobility of the preinitiation complex formed on the DHFR and AdML promoters shows that, as with the previous observation¹⁹, cyclin

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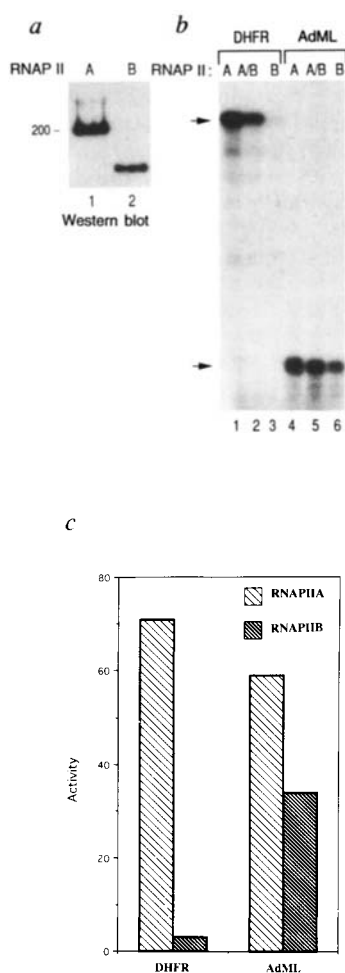
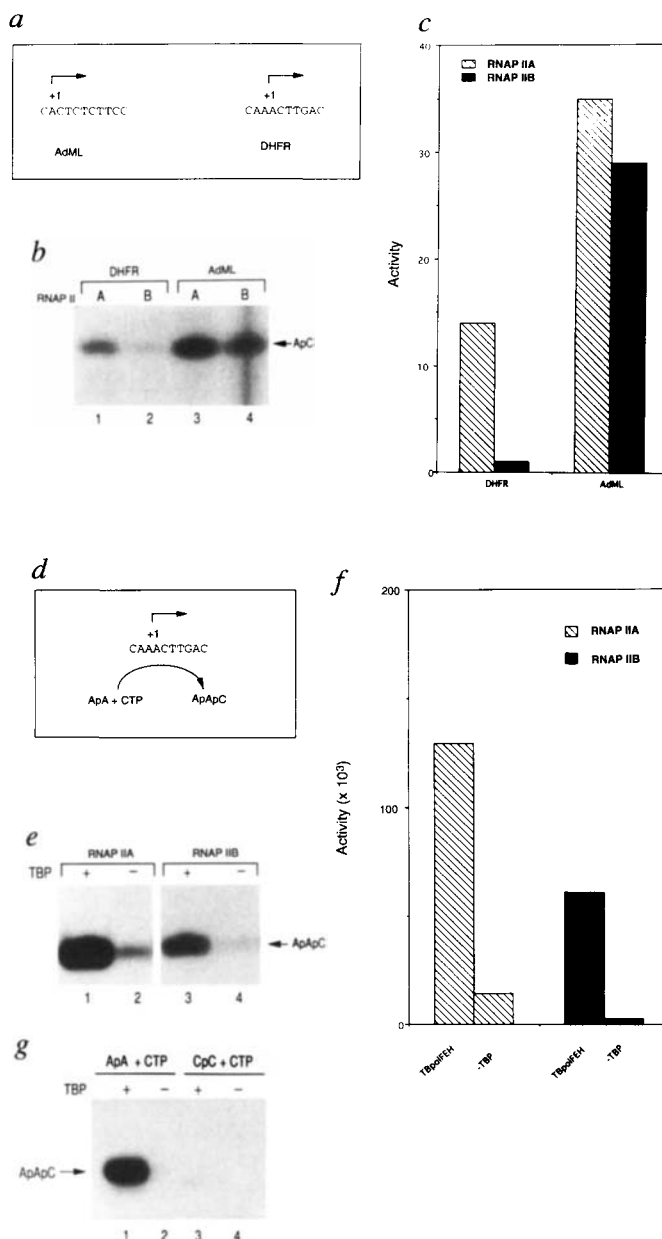


FIG. 2 Abortive initiation by A and B forms of RNAPII. **a**, ▶ Transcription start sites in the murine DHFR and AdML promoters. **b**, Dinucleotide synthesis on DHFR and AdML with different forms of RNAPII, as indicated. Reactions were performed as described²⁰ and under transcription conditions, but only ATP (500 μ M) and CTP (1 μ M) with 10 μ Ci [α -³²P]CTP were added. Products were treated with 1 unit of alkaline phosphatase (Boehringer) for 30 min at 37 °C and resolved on a 25% polyacrylamide-urea gel. **c**, Quantification of the data presented in **b** using a GS-250 Molecular Imager (Bio-Rad). **d**, Dinucleotide priming of abortive initiation on the murine DHFR promoter. **e**, Trinucleotide synthesis (ApApC) in the presence of ApA (1 mM) and [α -³²P]CTP by different forms of RNAPII, as indicated. dATP (100 μ M) was added to the reaction, as required for the activity of TFIIF²⁰ (P. Kumar and D.R., manuscript in preparation). Specificity of dinucleotide priming was monitored by omitting hrTBP from the preinitiation complex. Reactions were performed as in **b**. **f**, Quantification of the data presented in **e** using a GS-250 Molecular Imager (Bio-Rad). **g**, Trinucleotide synthesis in the presence of ApA (lanes 1 and 2) or CpC (lanes 3 and 4). Specificity of dinucleotide priming was also monitored by omitting hrTBP from the preinitiation complex (lanes 2 and 4). Reactions were performed as described for **e**, but ApA was replaced by CpC. The trinucleotides ApApC and CpCpC are present 19 and 39 times, respectively, in the plasmid containing the DHFR promoter.

FIG. 1 Transcription from the murine DHFR promoter in a reconstituted system with A and B forms of RNAPII. **a**, Western blot analysis of purified RNAPII A (lane 1) and B (lane 2) forms. Polyclonal antibodies against exon 5 of the largest subunit of RNAPII (a gift from R. Weinmann) were used. **b**, Products of transcription reactions containing the murine DHFR or AdML promoters with different forms of RNAPII as indicated. RNAPII was added at saturating amounts equivalent to 40 mU of promoter-independent transcriptional activity¹³. Transcription was assayed on a linear 390-bp G-less cassette for the DHFR promoter and on a 50-bp U-less cassette for the AdML promoter. **c**, Quantification of the results from **b**. Quantification was performed on GS-250 Molecular Imager (Bio-Rad).

METHODS. RNAPII A was purified as described⁷ with an additional immunoaffinity chromatography step using an anti-CTD column. RNAPII B was recovered in the flowthrough fraction. Transcription reactions were performed as previously described²⁶, and contained a linear plasmid DNA carrying the DHFR or AdML promoters (0.2 μ g in 40 μ l reaction volume) and recombinant human transcription factors (TBP, TFIIB, TFIIE and TFIIIF) and highly purified RNAPII (40 mU)⁴ and TFIIF (phenyl-Superose fraction)^{26,27}.



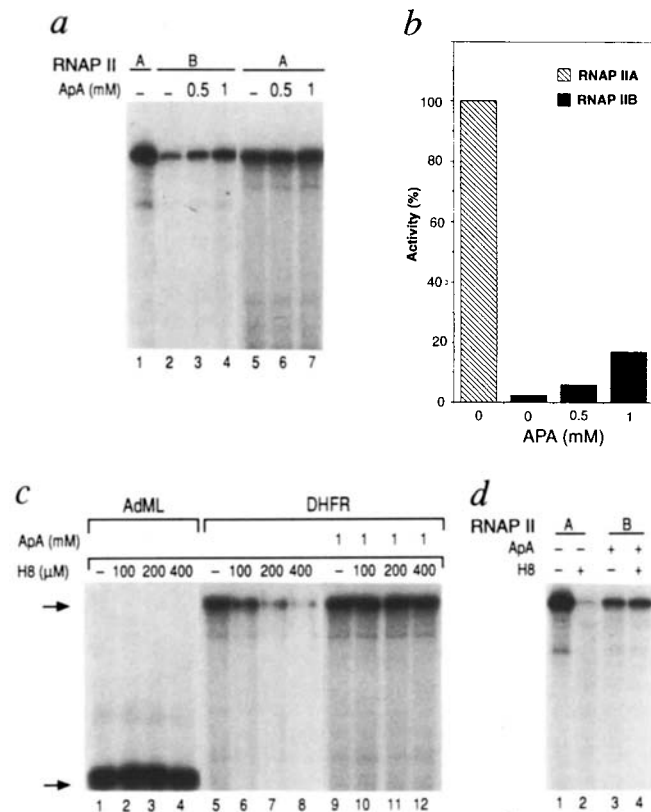
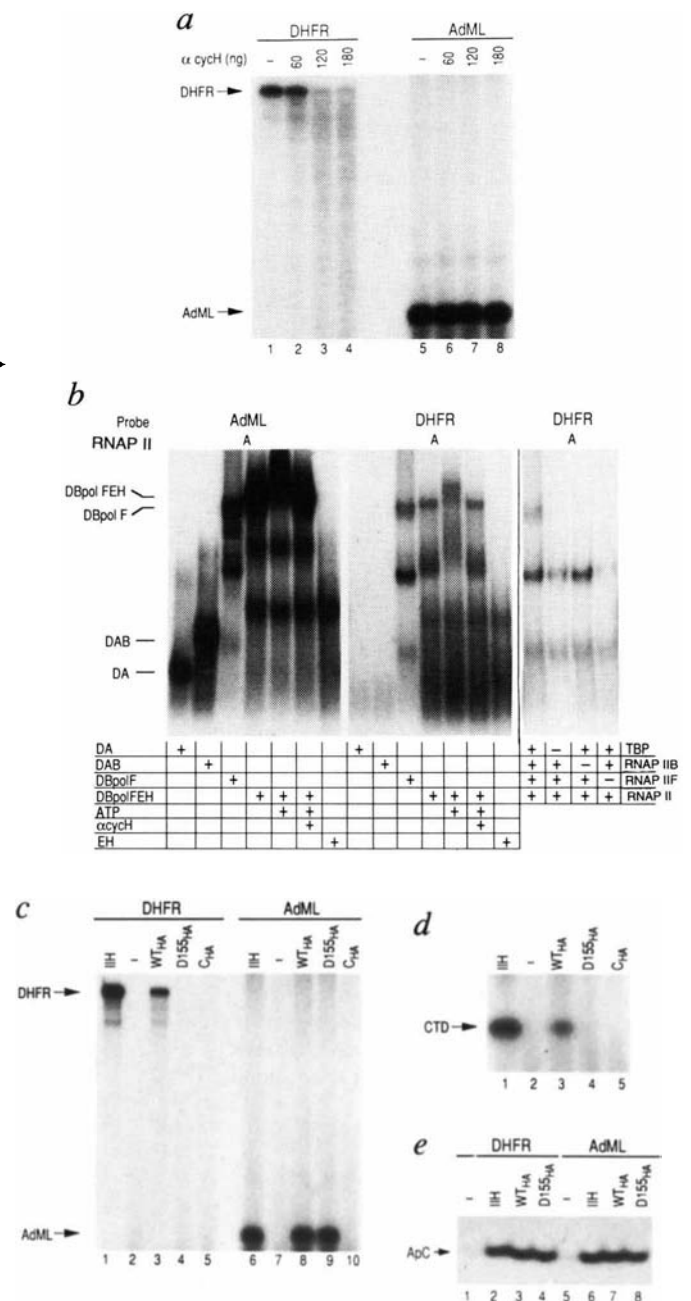


FIG. 3 **a**, Dinucleotide priming rescues run-off transcription of the *DHFR* promoter. Autoradiograph of a denaturing polyacrylamide gel containing the run-off products of *DHFR* transcription by the A and B forms of RNAPII as indicated. The preinitiation complex was formed in presence of ApA (as indicated) and transcription initiated by the addition of ribonucleoside triphosphates to a final concentration of 500 μ M. All other procedures were as described above. Autoradiography of the gel was overexposed to detect signals in lanes 2–4. **b**, Quantification with GS-250 Molecular Imager (Bio-Rad) of the data. **c**, Inhibition of *DHFR* transcription by H8, an ATP analogue. Reaction conditions were as described above with the following modifications: preinitiation complexes were formed for 30 min at 30 °C and preincubated with fresh dilutions of H8. Concentration of ribonucleoside triphosphates added was 100 μ M. Reactions contained RNAPIIA. In lanes 9–12 the preinitiation complexes were formed in the presence of ApA before the addition of ribonucleoside triphosphates. **d**, Primed transcription of the *DHFR* promoter by RNAPIIB is not susceptible to kinase inhibitor H8. H8 was applied at 400 μ M (lanes 2 and 4) to reconstituted *DHFR* transcription reactions performed with RNAPIIA (lanes 1 and 2) or RNAPIIB (lanes 3 and 4). ApA (1 mM) was added as indicated.

FIG. 4 **a**, Cyclin H antibodies¹⁹ inhibit transcription from the *DHFR* promoter. Autoradiograph of the run-off transcription from the *DHFR* and AdML promoters. Preinitiation complexes were formed for 30 min at 30 °C, followed by the addition of the indicated amounts of the cyclin H antibodies and further incubation for 30 min. Transcription was initiated by the addition of ribonucleoside triphosphates to the final concentration of 500 μ M. **b**, Autoradiograph of the binding assay on the *DHFR* and AdML promoters upon treatment with cyclin H antibodies. Binding was performed as described¹⁹ with the double-stranded oligonucleotide probes corresponding to either AdMLP (5'-TCCTGAAGGGGGGCTATAAAGGGGGTGGGGGCGCTTCCTCACTCTCTCCGCATCGCTGT-3') or the *DHFR* (5'-AAGCTGCGCAAGTGGTACACAGCTCAGGGCTGCGATTTCGCGCCAAACTTGAC-3') promoters. Complexes were formed in a 30-min incubation period at 30 °C, followed by the addition of the cyclin H antibodies. Samples were incubated for another 30 min before the addition of 1 mM ATP. Complexes were resolved by electrophoresis on a 4.5% native polyacrylamide gel in 1/2 $\times$ TBE at 40 mA. **c**, Immunoprecipitations of HA-tagged MO15/Cdk7, but not of the kinase-deficient mutant D155N, support transcription of the *DHFR* promoter. Transcription reactions were as described above, but immunoprecipitates of HA-tagged Cdk7 (lanes 3 and 8) or the kinase-deficient mutant D155N (lanes 4 and 9) were added in place of TFIIF (lanes 1 and 6) to transcription directed by the *DHFR* (lanes 1–5) and the AdML (lanes 6–10) promoters. Negative control was provided by omitting TFIIF (phenyl-Superose fraction), or using immunoprecipitates from cells transfected with the empty expression vector (lanes 2 and 7). The kinase-deficient mutant D155N has been previously described²⁵. Lysates were prepared as described²⁵ and precleared on protein-A beads and used for immunoprecipitation with HA-ascites. **d**, Kinase assay of the immunoprecipitates used in the transcription assay. Immunoprecipitates of HA-tagged Cdk7 (lane 3) and HA-tagged D155N (lane 4) were incubated with the CTD peptide in presence of [γ -³²P]ATP for 30 min at 30 °C, as described²⁵. Immunoprecipitates from cells transiently transfected with the empty vector were used as a negative control (lane 5). Positive control was provided by TFIIF (phenyl-Superose fraction) (lanes 1 and 2). **e**, Dinucleotide synthesis on *DHFR* (lanes 1–4) and AdML (lanes 5–8) promoters in presence of TFIIF (lanes 2 and 6), or the immunoprecipitates of HA-tagged Cdk7 (lanes 3 and 7) or the kinase-deficient HA-tagged mutant D155N (lanes 4 and 8). Reactions were done as described in Fig. 2b.



H antibodies suppress the shift associated with CTD phosphorylation (Fig. 4b).

To address the role of Cdk7 kinase activity in *DHFR* transcription directly, we prepared TFIID from cells transiently transfected with a haemagglutinin (HA)-tagged kinase-deficient mutant of Cdk7, as previously reported²⁵. HA-mediated immunoprecipitates of TFIID containing wild-type Cdk7 or kinase-deficient mutant D155N²⁵ were tested both for their ability to phosphorylate a CTD peptide and for their ability to support transcription in a TFIID-dependent manner. As shown in Fig. 4c, immunoprecipitates of both the wild-type and mutant (D155N) supported TFIID-dependent transcription on the AdML promoter (Fig. 4c, lanes 8 and 9). In contrast, the *DHFR* promoter only supported transcription with the immunoprecipitates containing wild-type Cdk7, but not the D155N mutant (Fig. 4c, lanes 3 and 4). As expected, the mutant form of Cdk7 was

unable to phosphorylate the CTD (Fig. 4d). To analyse further whether phosphorylation of the CTD was required for initiation, the above immunoprecipitates were analysed in abortive dinucleotide synthesis, as in Fig. 2b. High levels of abortive initiation were supported by immunoprecipitates of the wild-type and kinase-deficient mutant of Cdk7 (Fig. 4e). Thus, although the CTD has been implicated in initiation (see above), its phosphorylation by Cdk7 is not required.

Our studies indicate that the CTD is important for initiation of transcription of the TATA-less *DHFR* promoter, that is, the formation of the first phosphodiester bond. However, phosphorylation of the CTD by Cdk7, although essential for transcription of the *DHFR* promoter, is not required during initiation of transcription. This indicates that phosphorylation of the CTD by Cdk7 is important during the steps after initiation, probably promoter clearance or elongation. □

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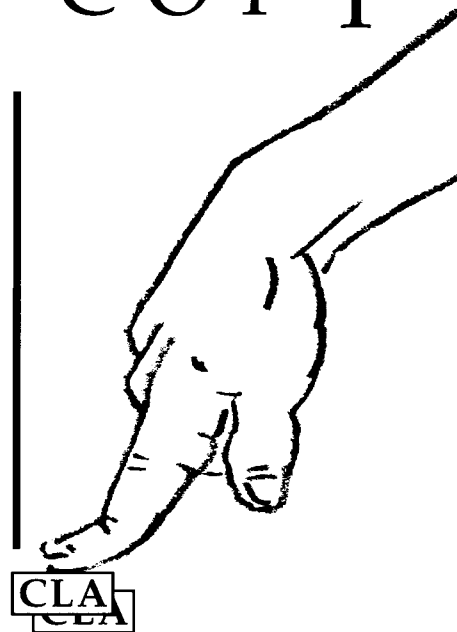
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